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(74) Agents: **BRUNO, Enrica et al.**; c/o Società Italiana
Brevetti S.p.A., Piazza di Pietra, 39, I-00186 Roma (IT).

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(71) Applicant (*for all designated States except US*): **CONSIGLIO NAZIONALE DELLE RICERCHE [IT/IT]**;
Piazzale Aldo Moro, 7, I-00185 Roma (IT).

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(72) Inventors; and

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(75) Inventors/Applicants (*for US only*): **GERACI,
Domenico** [IT/IT]; Piazza Boccaccio, 1, I-90144 Palermo
(IT). **COLOMBO, Paolo** [IT/IT]; Via Croce Rossa, 113,
I-90146 Palermo (IT). **DURO, Giovanni** [IT/IT]; Via
Principe di Paternò, 137, I-90145 Palermo (IT). **IZZO,
Vincenzo** [IT/IT]; Via Sacco e Vanzetti, 13, I-90121
Palermo (IT). **COSTA, Maria, Assunta** [IT/IT]; Via
Monti Iblei, 41, I-90144 Palermo (IT).

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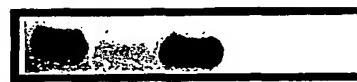
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(54) Title: PARIETARIA JUDAICA NS-LTP ANTIGEN VARIANTS, USES THEREOF AND COMPOSITIONS COMPRISING THEM

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1 2 3 4 5

A



IgE

B



IgG4

(57) Abstract: The present invention relates to hypoallergenic variants of ns-LTPs allergens, to pharmaceutical compositions comprising them and to the use of such variants for the preparation of medicaments suitable in the treatment and in the prevention of the allergic forms associated with an ns-LTP allergen, in particular to the allergen corresponding to the variant used.

PARIETARIA JUDAICA NS-LTP ANTIGEN VARIANTS, USES THEREOF AND
COMPOSITIONS COMPRISING THEM

DESCRIPTION

Field of the invention

5 The present invention relates to the fields of the prevention and the treatment of allergic symptoms associated with allergens belonging to the non-specific Lipid Transfer Protein (ns-LTPs) family.

State of the art

10 Ns-LTPs proteins are small proteic molecules of approximately 10 KDa that demonstrate high stability, and are naturally present in all vegetal organisms studied to date. In several species they have also been identified as allergens, as in the case of the Rosaceae Prunoideae 15 (peach, apricot, plum) and Pomoideae (apple), and Graminaceae, as in the Urticaceae like Parietaria Judaica (18-23).

20 These proteins are characterized by their ability to transport lipids through membranes *in vitro*, an ability justifying their denomination and corresponding to at least some of the activities exerted *in vivo* (17).

25 However, in spite of the different functions and of the heterogeneity of their sequence, ns-LTPs have a highly conserved secondary structure, comprising four alpha-helices (separated by loops) and one folded beta layer arranged in the 5'-3' direction according to a α - α - α - β pattern.

30 This structure is provided by the presence of four disulfide bridges formed by eight cysteine residues present in the 4 alpha-helices in the fourth loop, in the folded beta-layer and in the amino-terminal region (cfr. ref. 29).

In particular:

- a first disulfide bridge connects the amino-terminal region and the third alpha-helix,
- a second disulfide bridge connects the first alpha-helix and the third alpha-helix,

- a third disulfide bridge connects the second alpha-helix and the fourth loop, and

- a fourth disulfide bridge connects the third alpha-helix and the folded beta-layer.

5 These cysteine residues are highly conserved in all ns-LTPs, and with reference thereto a consensus sequence can be derived (17).

10 Despite their high conservation, given the sequence heterogeneity of ns-LTPs, no notation system for the residues forming disulfide bridges valid for all ns-LTPs exists, though those skilled in the art may easily single out such cysteines using the knowledge of the state of the art.

15 With particular reference to the Parj1 protein, and specifically to the mature ParJ1.0102 form, the cysteines apt to form disulfide bridges are the residues 4, 14, 29, 30, 50, 52, 75 and 91, and the related bridges are arranged in the order Cys4- Cys52 (first bridge), Cys14- Cys29 (second bridge), Cys30- Cys75 (third bridge), 20 Cys50- Cys91 (fourth bridge) (12).

25 Par J1, besides being an ns-LTP, represents, together with Par J2, one of the major allergens of the Parietaria Judaica (PJ), a plant whose pollen constitutes one of the most widespread environmental antigens, especially in the Mediterranean area (1).

In fact, Parietaria Judaica pollen contains at least nine allergens with molecular masses ranging from 10 to 80 KDa and different capabilities of binding IgE [1-9].

30 Thereamong, Parj1, in the two isoform par11.1.2 and Parj1.0201 isolated from independent genes (10) and Parj2 acquire a remarkable relevance as major allergens. In particular these two ns-LTPs are capable of inhibiting the majority of specific IgE against Parietaria allergens, and, upon administration, both have an immunological behaviour in all alike that of the commercial extracts commonly used (11).

However, ParJ1 and ParJ2 do not constitute the sole ns-LTPs having allergenic properties. Recently, some scientific papers describing the characterisation of new allergenic molecules homologous to the ns-LTPs have
5 been published (18-23).

Despite sequence heterogeneity, following cross-reactivity experiments between different ns-LTP allergens and related produced antibodies, it was demonstrated that ns-LTP constitute a widespread family of allergens (pan-allergen) as already described for profilin (19).
10

However, in comparison with the abundant information on the structure of this 'pan-allergen', exhaustive information on the localisation of the epitopes for IgE and IgG therein, as well as in the individual ns-LTP allergens (ParJ1 and ParJ2 included) are not available
15 (11, 12).

As a result of the mechanism in charge of the development of the allergic response, and of the verified role of IgG and IgE therein, the derivation of such a map would be instead of enormous relevance for the drafting
20 of a novel therapeutic approach to these allergic forms (13).

In particular, the derivation of molecules with reduced or even absent IgE binding capability, yet concomitantly capable of inducing IgG response, and in particular of IgG4, might be a landmark both from a therapeutic and a preventive point of view.
25

Such a molecule would allow immunosuppression of the T cell response with reduced or even absent side effects.

30 In fact, to date the therapy of an undergoing allergy consists in the mere pharmacological cure of the allergic symptomatology.

A preventive therapy represented by the specific immunotherapy (SIT) actually consists in the subcutaneous administration of diluted quantities of allergen to the patient so as to suppress the specific reaction towards
35 the allergen.

The majority of the commercial protein extracts used therefor however, are anyhow crude extracts, mixtures of several components in which a precise standardization of the allergenic component is 5 difficult.

Thus, the SIT strategy can entail the administration of allergenic components towards which the patient is not sensitive, inducing the secretion of IgEs specific towards other components of the extract. Moreover, the 10 administration of the total allergen entails the possibility of side effects which, though with extremely low occurrence, could even cause anaphylactic shock.

Concerning in particular the Parietaria Judaica, epidemiological studies have also highlighted a different 15 distribution of the two major allergens in the human population (12 millions of affected subjects in the Mediterranean area) where, approximately 20% of the PJ allergic patients do not exhibit a concomitant presence of IgE specific against both allergens. Therefore, an 20 administration of total or partially purified crude extracts could entail an administration of major allergens to which the patient is not allergic.

Hence, the use of recombinant molecules, allowing a 25 patient customized diagnosis and therapy, could represent a valid alternative to the traditional use of crude extracts.

In particular, the characterization and the development of alternative molecules with reduced side 30 effects, i.e., having a reduced or absent interaction with the IgE while maintaining the capability of binding the IgGs (in particular the IgG4) with respect to the wild type and therefore the capability of immunosuppressing the T response, could allow to implement an alternative approach overcoming the 35 disadvantages inherent to the traditional approach.

Such an alternative molecule with reduced anaphylactic capacity were in fact sought by producing

crude formaldehyde- or glutaraldehyde- polymerised extracts (16).

Although effective, as demonstrated by clinical trials, these modified molecules have proved however to 5 present the abovedescribed disadvantage of a difficult standardization of the extracts.

Following the advent of genetic engineering both recombinant allergens immunologically similar to the native allergens (14 and 15), and recombinant allergen 10 having instead a reduced allergenic activity with respect to the allergen wild type (therefore therapeutically suitable as a substitute of the latter), have been derived in a pure form.

None of such a mutant have not however been derived 15 with particular reference to the ns-LTPs allergens.

SUMMARY OF THE INVENTION

An object of the present invention is a variant of an allergen belonging to the ns-LTP protein family, specifically a hypoallergenic variant of a complete 20 allergen or fragment thereof of the ns-LTD protein family.

In particular object of the invention is a variant of an allergen belonging to the ns-LTP family which lacks at least one of the four disulfide bridges constituting 25 the structure of said allergen.

A first advantage of the variant of the present invention is that with respect to the native allergen it has a reduced or even absent capability of binding IgE, having concomitantly an intact capability of binding IgG 30 of the said subjects.

This differential binding capability is particularly enhanced in the variants wherein such a missing bridge be localized in the amino-terminal region of the allergen at the domain alpha-helix 1- loop 1- alpha-helix 2, as they 35 have a particularly reduced IgE binding activity, especially in the variants lacking at least two disulfide bridges.

In case such a variant be lacking three, or all four, of the disulfide bridges of the native allergen, the relevant IgE binding activity is reduced up to be substantially absent. Such a variant constitutes accordingly a preferred embodiment of the invention.

The relevance of such a differential binding capability of the variant of the invention lies in that according to the role of the two immunoglobulins in the molecular mechanism of the allergic response evidenced in the above paragraph, it turns out in molecules, that although immunogenic have a reduced or absent allergenicity.

Variants of the invention which mainly maintain most of amino acid sequence of the wild type allergen, and has accordingly substantially the same length of the said allergen, constitute in this connection a preferred embodiment of the invention.

In particular variants consisting of a mitein in which at least one of the cysteines constituting said disulfide bridges is deleted, or substituted with an amino acid residue not capable of forming disulfide bridges, are preferred.

In this latter case, the substitution of the cysteine residue with serine or alanine, as amino acids tested compatible with the ns-LTP α - α - α - α - β structure, proved particularly effective.

The embodiment related to variants of the major allergens of Parietaria Judaica is particularly preferred.

In particular, the Parj1 variants, specifically the Parj1 miteins in which the deleted or substituted residues are the cysteines 4, 14, 29, 30, 50, 52, 75 and 91, and in particular the variants having a sequence selected in the group comprising the sequences reported in the sequence listing as SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 10, are especially relevant.

Object of the present invention is also a polynucleotide coding for the variants of the present invention, in particular for the above indicated muteins, and specifically the polynucleotides comprising a sequence selected in the group comprising the sequences reported in the sequence listing as SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7 and SEQ ID NO: 9, as well as the vectors comprising them.

In the light of what set forth above, object of the present invention are also any of the above mentioned variants for use as medicament or as a diagnostic agent, and in particular for use in the treatment and/or the prevention and/or the diagnosis of the allergic form associated with an allergen belonging to the family of ns-LTP proteins, (this in light of the pan-allergen characteristic verified for the ns-LTP allergens), in particular to the allergen corresponding to said variant.

In the specific case, hereto disclosed by way of example, of major allergens of Parietaria Judaica showing an uneven ability to stimulate serum IgE production in allergic patients, a specific diagnosis for the individual allergen may be attained using the variants of the invention, e.g., as follows: initially the recombinant version of each native molecule (parJ1 and Parj2) is used for a specific diagnosis of the allergy by skin prick test. Patients sensitive to one of two allergens can then be analyzed for positiveness to the individual allergen variants to which they tested positive in order to highlight negatively testing variants. Then, such variants can be administered in substitution of commercial protein extracts developing an allergen-specific immunotherapy. Further modes of employ for diagnostic as well as therapeutic ends are anyhow derivable by those skilled in the art in light of the knowledge of the state of the art.

Object of the present invention is also a pharmaceutical composition comprising a therapeutically

effective quantity of at least one of the variants, or a polynucleotide or a vector among the above mentioned ones, and a pharmaceutically acceptable carrier, as well as all the matter compositions comprising at least one of
5 the above mentioned molecules and one carrier chemically compatible therewith.

This pharmaceutically and/or chemically acceptable carrier can be any one carrier known to the art as suitable in pharmaceutical or matter compositions
10 containing the molecules like the above mentioned ones, therefore in particular peptides and conjugates and/or oligonucleotides in any form, in particular in solid and in liquid form; an example of composition in liquid form is provided by compositions whose carrier is water,
15 saline solutions, like, e.g., solutions containing NaCl and/or fosfate, or other solutions containing buffer molecules.

A still further object of the present invention is a kit for the derivation of a subject-customised allergogram, for an allergic form associated with an ns-LTP allergen comprising

- a first composition comprising said ns-LTP allergen in native form together with a chemically and/or pharmaceutically acceptable carrier;
- at least one composition comprising a single variant of said ns-LTP allergen as abovedescribed and a chemically and/or pharmaceutically acceptable carrier;

said allergogram being derivable contacting said compositions with immunoglobulins of said subject and observing the effects thus obtained.

Particularly preferred are the embodiments in which the kit comprises, besides said first composition, a number of compositions each comprising a single variant of said ns-LTP allergen, equal to the number of variants of said ns-LTPs allergen and that in which said allergen is a Parietaria Judaica allergen, specifically ParJ1 (in any one form thereof) or ParJ2 (in any one form thereof).

In particular such compositions can be contacted with immunoglobulins by 'skin prick test' *in vivo*, or on patient's tissues like, e.g., blood, *in vitro*. Other modes of employ of the kit of the present invention to diagnostic ends are derivable by those skilled in the art in light of the knowledge of the state of the art. The invention will be better described with the aid of the attached figures.

DESCRIPTION OF THE FIGURES

Fig. 1 reports the amino acid sequences of the native Par J 1.0102 and Par j 2.0101 aligned therebetween and with respect to the three-dimensional structure thereof. The notation of the amino acids relates to the sequence of the Par j 1.0102.

Fig. 2 shows the amino acid sequences of the Par j 1.0101 and of some ns-LTP proteins aligned thereamong. The arrows indicate the disulfide bridges present in the three-dimensional structure of the proteins. The amino acids are indicated in one-letter code. The Cs reported in the last row of the table indicate the cysteine residues conserved in all proteins of the ns-LTP family.

Fig. 3 reports the schematic representation of the mutants of the major allergen of the Parietaria Judaica Par j 1.0102, the sequence thereof being reported in the sequence listing as SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 10. The amino acids are reported in one-letter code. The underlined amino acids indicate the mutations effected in the native sequence. The arrows indicate the disulfide bridges.

Fig. 4 reports in panel A the Western blot analysis results showing the IgE binding activity of the rParj1 and its disulfide bond variants by using a pool of sera (n=30) from monosensitive Pj allergic patients.

Panel B shows a Coomassie Brilliant Blue staining of the recombinant proteins used.

In both panels on the first lane the result referred to the native Par j 1.0101 is reported; on the second

lane the result referred to the mutant PjA is reported; on the third lane the result referred to the mutant PjB is reported; on the fourth lane the result referred to the mutant PjC is reported; and on the fifth lane the result referred to the mutant PjD is reported.

Fig. 5 shows in panel A the outcome of a Western Blot analysis on a pool of sera from PJ allergic patients, aimed at demonstrating the IgE binding capability (activity) of some mutants of the present invention extensively disclosed in example 3.

On the first lane the result referred to the native Par j 1.0101 is reported; on the second lane the result referred to the mutant PjA is reported; on the third lane the result referred to the mutant PjB is reported; on the fourth lane the result referred to the mutant PjC is reported; and on the fifth lane the result referred to the mutant PjD is reported.

In panel B the outcome of a Western blot analysis on a pool of sera from PJ allergic patients, aimed at demonstrating the IgG4 binding capability of the same abovedescribed mutants, it also extensively described in example 3, is shown.

On the first lane the result referred to the native Par j 1.0101 is reported; on the second lane the result referred to the mutant PjA is reported; on the third lane the result referred to the mutant PjB is reported; on the fourth lane the result referred to the mutant PjC is reported; and on the fifth lane the result referred to the mutant PjD is reported.

Fig. 6 reports an histogram showing the results of the ELISA detection experiment carried out using the pool of sera used in example 3, extensively described on the example 4. Black histogram indicate results obtained with allergic sera; dotted square indicated results obtained with non allergic serum. On the y axis the optical density measured and in the x axis the proteins tested (the rParj1 and its disulfide bond variants PjA, PjB,

PjC, and PjD)) are reported.

Figure 7 shows ten diagram reporting the results of the ELISA detection carried out using monosensitive sera from ten Pj allergic patients extensively described on example 4, each diagram corresponding to a respective patient.

On each diagram on the y axis the optical density measured and in the x axis the proteins used (the rParj1 and its disulfide bond variants PjA, PjB, PjC, and PjD)) are reported.

On each diagram black squares indicate allergic sera, white squares a non allergic serum.

Fig. 8 reports an histogram showing the results of the ELISA detection of the Ig binding activity of a rabbit polyclonal immune and pre-immune antisera against rParj1, extensively described on the example 6.

On the y axis the optical density measured and in the x axis the antigens used (the rParj1 and its disulfide bond variants PjA, PjB, PjC, and PjD)) are reported.

Black squares indicate results obtained on rabbit immune, checkered squares results obtained on rabbit preimmune.

DETAILED DESCRIPTION OF THE INVENTION

The experimental approach that led to the present invention consisted of a mutagenesis strategy aimed at the targeted disruption of all the disulfide bridges present in all the ns-LTPs. This in order to generate molecules with reduced or absent affinity to IgE, yet with intact affinity to the other classes of antibodies apt to compete with the specific IgEs against the native allergen. The ns-LTPs allergen ParJ1, whose sequence and structure is reported in comparison with Par J2 in figure 1, and in particular the isoform Par j 1.0102 (the primary sequence thereof being reported in the annexed sequence listing as SEQ ID NOS:1 and 2), was taken as a molecular model, to be used for carrying out mutagenesis

and the subsequent verification of the properties of the obtained mutant. In particular, recombinant DNA technology was resorted to in a strategy of site-directed mutagenesis against cysteines 4, 29, 30, 5 50 and 52, i.e., the cysteines constituting the disulfide bridges according to the structural model known to the art.

The results of these experiments demonstrate the close relationship existing between the three-dimensional structure of the protein and the capability of forming epitopes for the IgEs, and in particular that the gradual disruption of the disulfide bridges causes a reduction of the serum IgEs binding activity thereof, whereas it does not affect the IgG4 binding activity thereof.

This in light of the Western blot analysis described in example 3, and in figure 5, in which lanes 2, 3 and 4 (see Fig. 5 panel A) show the reduction of the serum IgEs binding activity by the mutants of the present invention, which should be construed as three-dimensional mutants.

In particular, the Cys29-Cys30 mutant (PjA) shows a very weak binding band (Fig. 5 lane 2) whereas the Cys50-Cys52 mutant (PjB) is somehow still capable of binding the IgEs (Fig. 5, lane 3). Instead, more remarkable is the result shown by the PjC and PjD mutants (Fig. 5, lanes 4 and 5) for which no binding to human IgEs can be highlighted.

Such results have been confirmed by ELISA and IgE inhibition assays (see examples 4 and 5) where the PjB was the only variant still able to bind Parj1-specific IgE antibodies in solution while the other variants exhibited a very low inhibition capacity. The loss of additional disulfide bridges (PjC and PjD) leads to the absence of any IgE recognition (see Example 4 and Figure 4).

These results all together show that ns-LTP Parj1 variants lacking of at least one disulfide bridge have a reduced allergenicity, which is even absent in the

variants wherein the lacking bridge is localized in the aminoterminal region of the allergen at the domain alpha-helix1-loop1-alphahelix2, in particular when the lacking bridges are at least two.

5 The maintenance by these variants of an overall antigenicity that, notwithstanding the reduced or absent allergenicity, is comparable or identical to the one of the wild type allergen, has been shown by experiments
10 wherein binding activity of wild-type allergen and its variants to antibodies different than IgE, have been compared.

15 Such experiments are Western Blots carried out using as antibodies rabbit polyclonal antibodies and IgG4 of Pj allergic patients, extensively described in examples 6 and 3 respectively.

20 In these experiments the hypoallergenic variants generated by genetic engineering presented a similar behavior compared to the wild type, with a low reduction of their binding activity towards the anti-rParj1 rabbit antibodies.

25 Accordingly, a variant that lacks of at least one disulfide bridges still contains several protein domains similar to the native molecule and, although at different extent, is apt to induce the production of IgG antibodies (see example 3 and 6). IgE production and/or IgE-mediated presentation of the allergen, would be prevented by such "blocking" antibodies and reducing T cell proliferation and release of cytokines (25).

30 The above data have been confirmed also *in vivo* in particular by Skin Prick Test (SPT) analysis as described in example 7, where the pure recombinant proteins were tested on ten PJ allergic patients (the same analyzed by ELISA in example 4 and figure 7).

35 With regard to the single mutants, PjA showed a very low IgE binding activity and only 3 out of 10 patients with cutaneous Type I hypersensitivity and a reduced wheal area respect to that one induced by wild-type

allergen. On the contrary, loss of the Cys50-Cys91 and Cys4-Cys52 bridges seems to have a minor effect since an IgE binding activity and a positive SPT are still present. The loss of additional disulfide bridges (PjC and PjD) leads to the absence of any cutaneous reaction (see example 7).

These results obtained analyzing individuals demonstrate the reliability of the above described data *in vitro*, and above all that while the disruption of the disulfide bridges in the amino terminal region (in the specific case Cys4-Cys52 and Cys50-Cys91) affect even if not markedly the human IgE binding capability of this mutant, the disruption of all the four bridges (in the specific case Cys4-Cys52, Cys14-Cys29, Cys30-Cys75 and Cys50-Cys91) have a devastating effect on the IgE recognition, and therefore on the allergenic response.

Accordingly, concerning the development of therapeutically useful hypoallergenic molecules, variants lacking of three or four bridges, as in the specific case PjC and PjD mutants, are considered as preferred embodiments. The above was demonstrated using a pool of sera as well as a cohort of individual patients, indicating that the obtained result is representative of the immune response of the allergic population.

It is pointed out that although obtained using specific variants derived by mutating the wild type allergen, these results are in fact not limited to the said specific variants, neither to the techniques used for the relevant derivation.

As such results are consequent to the modification of the three-dimensional structure of the allergen, they could anyhow have been obtained by any mutagenesis allowing the disruption of the disulfide bridges.

Accordingly any variant obtainable by deletion, substitution and/or the insertion of one or more amino acidic residue which results in variants lacking of at

least one disulfide bridge is included in the object of the invention.

The strategy of point mutation has however the remarkable advantage of allowing the insertion of minimal variations at the level of the primary sequence of the native protein and therefore of generating mutants that are more likely not to interfere with the variant recognition operated by the T cells, and above all the possibility to generate proteins having a high reproducibility.

Variants having substantially the same length of the wild type are accordingly considered preferred.

With regard to the techniques used for deriving the variants of the invention, it is not limited to the genetic engineering ones, as they are obtainable by techniques like chemical mutagenesis (formaldhehyde and gluteraldhehyde) which allow the disruption of disulfide bridges even in absence of mutations.

The genetic mutagenesis imply however the remarkable advantage of allowing the generation of proteins having a high reproducibility, while the, chemical mutants do not ensure a denaturation pattern constant at every preparation.

Furthermore, as the strategy described herein is independent on the epitope sequence on itself since it is based on the modification of the three-dimensional structure of the IgE determinants, the adopted mutagenesis strategy is actually independent from the primary sequence of the allergen (and therefore from the sequence of the specific IgE epitopes).

For this reason variants of all the proteins with allergenic activity belonging to the ns-LTP family (including Parj2), are included in the object of the invention due to the conserved structure (cfr. e. g.s figure 2 wherein Par j1 sequence is reported in comparison with the sequence of other ns-LTPs together with the placement of the disulfides bridges):

In particular variant of the invention is not only any other mutein of the ParJ1 allergen or of other ns-LTP allergens which, independently from the mutation carried out (substitution and/or deletion of one or more amino acid residues) and of the way in which such a mutation is carried out (e.g., by the above mentioned techniques), retain a structure equivalent to that of the corresponding native allergen lacking at least one disulfide bridge.

Thus the disruption of the disulfide bridges in ns-LTP allergens underlies *per se* a limited or absent IgE binding ability of patients allergic to the related variants..

Moreover, in particular in light to what is known in the art concerning the high conservation of the structure and the cross-reactivity that have led to the singling out of the so-called ns-LTP pan-allergen (see above) these data are indicative not merely of a suitability in the therapy and prevention of the allergic forms caused by the allergens corresponding to the individual variants, but also in the therapy and prevention of allergic forms caused by ns-LTP allergens other than those corresponding to the variants used.

A person skilled in the art can derive on the basis of his knowledge any information suitable for deriving uses, compositions and kit described in the summary of the invention.

With the aid of the following examples, a more detailed description of specific embodiments will now be given, in order to give a better understanding of the objects, characteristics, advantages and operating methods of the present invention.

EXAMPLES

Example 1: Cloning and Expression of Par J 1.0102

For the production of the major allergen of Parietaria Judaica Par j 1.0102 the pQE30 prokaryotic vector (Qiagen) was used. The latter characteristically

expresses recombinant proteins fused to a short histidine tail and inducible with isopropyl- β -D-thiogalactoside (IPTG). The histidine residues allow the purification of the recombinant protein by affinity chromatography.

For this reason, 1 ng of the P5 clone containing the processed version of the Par j 1.0102 (EMBL accession number X77414), the sequence thereof being reported in the annexed sequence listing as SEQ ID NO: 12, was subjected to 30 cycles of polymerase chain reaction (PCR) amplification at the following design: 94°C for 1 min, 52°C for 1 min, 72°C for 1 min. The synthetic primer oligonucleotides P5 forward and P5 reverse, the sequence thereof being reported in the annexed sequence listing as SEQ ID NO:11 and SEQ ID NO: 12, respectively, were used.

The fragment thus generated was fractionated on 1% agarose gel in 1 X TBE, extracted, purified and digested with Bam H1 and Hind III restriction enzymes and cloned in the pQE30 VECTOR (Quiagen) previously digested with the same enzyme. The linearized vector and the digested fragments were incubated for 4 hours at 16° C in presence of the enzyme DNA ligase according to different stoichiometric ratios. The reaction mixture was then transformed in the bacterial strain M15. The recombinant clones were sequenced with the method of Sanger and the nucleotide sequence thus determined demonstrated that the DNA fragment inserted into the pQE30 vector was identical to that known in the art (10).

Example 2: Cloning and expression of conformational mutants of ParJ 1.0102

PjA mutant (Cys29→Ser and Cys30→Ser) was generated using the Transformer Site-Directed Mutagenesis kit (Clontech) following the manufacturer's instruction and using the oligonucleotide P5 (29,30) reported in the sequence listing as SEQ ID NO: 13 (mapping from nucleotide 88 to nucleotide 105) and the Parj1 sequence as a template. PjB mutant (Cys50→Ser and Cys52→Ser) was

generated by PCR using as primers the oligonucleotide P5(50-52) reported in the sequence listing as SEQ ID NO: 14 (mapping from nucleotide 91 to nucleotide 165) and P5 reverse oligonucleotide and 1 ng of the Parj1 clone as a template. The PCR fragment was digested with Pst I and Hind III restriction enzymes and ligated with the Pst I-Hind III linearized plasmid vector containing the Parj1 sequence (expressing the first 31 amino acids of the wild type Par j 1.0102 allergen). PjC mutant (Cys4→Ser, Cys29→Ser and Cys30→Ser) was generated by PCR amplification using the PjA variant as a template. The cysteine residue at position 4 was mutated by PCR using the oligonucleotides P5(triple), the sequence thereof being reported as SEQ ID NO: 15, and P5 reverse.

After purification, PCR fragment was digested with Bam HI and Hind III enzymes and cloned in the pQE30 vector previously digested with the same restriction enzymes. PjD mutant (Cys29→Ser, Cys30→Ser, Cys50→Ser and Cys52→Ser) was generated using the Transformer Site-Directed Mutagenesis kit (Clontech) following the manufacturer's instruction and using the synthetic oligonucleotide P5 (29,30) reported in the sequence listing as SEQ ID NO: 13 and the PjB variant as a template. All clones were sequenced with the method of Sanger (24) and the mutations and the open reading frames confirmed (See Fig.3 for details).

With this process 4 independent mutants, hereinafter designated PjA (SEQ ID NO: 3 and SEQ ID NO: 4); PjB (SEQ ID NO: 5 and SEQ ID NO: 6), PjC (SEQ ID NO: 7 and SEQ ID NO: 8), and PjD (SEQ ID NO: 9 and SEQ ID NO: 10) were isolated.

Example 3: Purification of recombinant proteins evaluation of the relevant capability of binding IgE of allergic patients

35 10 ml O/N culture of the recombinant clones (NM15 strain, Quiagen) were then used for an inoculation in 400 ml of 2YT broth (Bacto-tryptone 16 gr/l, Bacto-yeast 10

gr/l, NaCl 5 gr/l, pH 7,0) containing ampicillin and kanamycin at a final concentration of 100 µgr/ml and 10 µgr/ml, respectively.

A 1:40 dilution was grown for 1 hour at 37°C and, 5 after that, induced with 1 mM isopropylthio- β -galactoside for 4 hours at 37° C. Cells were harvested by centrifugation and the recombinant proteins purified by using the His Trap kit (Pharmacia) following the manufacturer's instructions. Recombinant proteins, 10 binding the HiTrap chelating column, were eluted using a buffer containing: 20mM phosphate buffer pH7.4. 0,5 M NaCl, 8 M UREA and 500 mM imidazole; fractions were analysed by 16% SDS-PAGE and Coomassie Brilliant Blue staining. Fractions containing the purified protein were 15 then diluted 1:100 in a buffer containing 20mM phosphate buffer pH7.4. 0,5 M NaCl and 20 mM imidazole to allow refolding of the protein, reloaded on the His Trap column and eluted with a buffer with no denaturing agents (20mM phosphate buffer pH7.4. 0,5 M NaCl and 500 mM imidazole). 20 Recombinant proteins were then desalted using a centrifugal filter device (Centriprep, Millipore) and analysed for their capability of binding human IgE from Pj allergic patients by Western blot as previously described (12), using a pool of sera (n=30) of Pj 25 allergic patients which did not receive any specific immunotherapy.

This analysis showed that the PjB mutant was still capable of binding human IgE while the PjA mutant retains only a weak IgE binding activity. The PjC and PjD mutants 30 did not show any IgE binding activity suggesting that the IgE recognition was dependent on the three-dimensional folding of the protein (Fig.4 Panel A).

After that, membranes were stripped and reprobed 35 with a His-tag specific reagent (INDIA™ Hisprobe-HRP, Pierce, USA) to check that the IgE-allergen complex was specific for the recombinant fused proteins. The concentration of the recombinant proteins was determined

20

by densitometric analysis of SDS-PAGE gels stained with Coomassie Brilliant Blue (see figure 4 panel B).

As a confirmation of this experiment another Western blot carried out using IgE and IgG4 of allergic patients.

5 Then, the proteins purified were fractionated on 16% PAGE-SDS and transferred on nitro-cellulose thanks to a Dry-blot system (Millipore). The membrane was incubated for 12-14 hours with a pool of sera from Pj allergic patients (1:5 dilution) in PBS-tween. The protein-human
10 IgE and IgG4 binding complexes are highlighted using respectively a secondary anti-IgE and anti-IgG4 antibodies conjugated to radish peroxidase. Thus, the complexes are highlighted using a chemiluminescence system (Super-signal, Pierce). The relevant results are
15 reported in figure 4.

Example 4: Elisa detection

20 The same pool of allergic sera from non-sensitive PJ allergic patients used in example 3 has been used in an ELISA experiment, showing the IgE binding activity of the rParj1 and its disulfide bond variants. A non allergic subject has been tested as a negative control on the ELISA.

25 The results confirm the pattern of reaction of the experiment of the example 3 (Fig.4 Panel A) with the PjB variant reacting in a way comparable to the wild-type allergen. A non allergic serum is shown as a negative control (Fig.6).

30 The IgE binding activity of the four Parj1 disulfide bond variants was also tested by ELISA using sera from ten monosensitive Pj allergic patients. Analysis of single sera showed a remarkable homogeneity of the reaction. In particular, the Cys4-Cys52 and Cys50-Cys91 bridges did not influence the allergenicity of the protein since this mutant (PjB) showed an IgE binding activity comparable to the wild-type allergen. On the other hand, the Cys14-Cys29 and Cys30-Cys75 bridges seem
35 to be crucial for the IgE recognition. All the variants

lacking those two bonds (PjA, PjC and PjD) presented low or even absent IgE binding activity. (Fig.7)

ELISA detection has been performed by adding 200 µl of a solution containing 5 µg/ml of antigen in coating buffer (sodium carbonate buffer pH 9,5) to each well of polystyrene plates overnight at room temperature. After several washing steps (1XPBS, 0,1% Tween 20) plates were saturated with a solution containing 5% BSA, 0,5% Tween 20 in coating buffer. After washing, 200 µl of serum (1:5 dilution) from Pj allergic patients or from a non allergic subject were incubated for 4 hours at room temperature. Bound IgE antibodies were detected with a goat antihuman IgE-HRP conjugate (Biosource International) diluted at a concentration of 0,5 ng/ml in 1XPBS, 0,25% BSA, 0,1% Tween 20 for 1 hour at room temperature. After several washes, colorimetric reaction was developed by adding 0,2 ml/well of substrate solution (0,4 mg/ml o-phenylenediamine in 0,1 M citrate buffer). Optical density was read at 495 nm in a BIO-RAD microplate reader.

Example 5: IgE inhibition assay

In order to investigate whether the disulfide bond variants were able to inhibit the binding of the IgE to the rParj1, increasing amount of each recombinant mutant were incubated with a pool of sera (n=10) of Pj monosensitive allergic patients.

The ability of the Parj1 disulfide variants to interact with IgE antibodies was determined by an ELISA inhibition experiment. A pool of sera (1:5 dilution) from ten monosensitive Pj allergic patients was preincubated overnight with increasing concentration of each disulfide bond variant (0,25-20 µg/ml of protein). The solutions were added to the ELISA wells coated with 5mg/ml of rParj1 and the ELISA steps were performed as above described. Percentage of inhibition was calculated according to the formula: % = 100-OD_A/OD_BX100, where OD_A

and OD_s represent the optical density read with the inhibited and non-inhibited pool of sera respectively.

The results are reported in the following Table I

5

Table I: Inhibition of IgE binding

Protein tested	%inhibition
rParj1	95%
PjA	16%
PjB	85%
PjC	14%
PjD	15%

The data reported in Table I suggests that all the variants lacking, at least, Cys14-Cys29 and Cys30-Cys75 disulfide bonds exhibit a comparable low level of inhibition (about 15%). On the contrary, the PjB variant (Cys50→Ser and Cys52→Ser) showed a high percentage of inhibition retaining a substantial ability of binding human IgE (about 85%).

15

Example 6: Rabbit polyclonal binding activity

Rabbits were immunized by PRIMM srl (Milan, Italy) using the rParj1 allergen. As a control, rabbit polyclonal antibodies were analysed on a Western blot using a *Parietaria judaica* crude extract detecting a band of about 14000 Da corresponding to Parj1 native molecular weight. ELISA plates were coated at the same conditions as above described, with the wild-type Parj1 and with equal amount of each recombinant disulfide bond variant, were probed with an anti-rParj1 specific polyclonal serum to analyse their binding activity.

25

Rabbit preimmune and immune sera were diluted at a concentration of 6 ng/ml and 200 µl of these solutions were incubated at room temperature for 1 hour. Wells were washed three times in 1XPBS, 0,1% Tween 20 and bound antibodies were detected using a donkey antirabbit Ig HRP

30

linked (Amersham) at a 1:1000 dilution. Colorimetric reaction and optical density were performed as above described.

5 The data obtained suggest that the PjA, PjB and PjC variants show a similar behavior exhibiting a slight reduction of their binding ability (about 10%) compared to the Parj1 binding. The PjD variant showed a reduced binding activity (about 20%) while the preimmune serum did not show any reactivity towards the proteins (Fig.8).

10 Example 7: Skin Prick test experiments with purified miteins

Ten patients, with a clear history of *Parietaria judaica* allergy and with skin prick test (SPT) monosensitivity to Pj commercial extract, were analysed 15 in this study. All the patients did not receive immunotherapy against Pj pollen and were not receiving glucocorticosteroid treatment. Allergens were used at 1 μ g/ml concentration diluted in 0,9% NaCl. About 20 μ l of the test solution was placed on the forearms at a 20 distance of more than 2.5 cm between each prick. All tests were performed in duplicate. Histamine was used as positive control and 0,9% NaCl solution as a negative control. Reactions were measured after 20 min. By comparison with the wheal area generated by histamine 25 (100%), positive SPT were divided in three classes: 4+ were assigned to SPT with an area \geq 100% of area induced by histamine; 3+ were assigned to an area \geq 80-100% and 2+ to an area \geq 50-80%. Two non-allergic patients (P.C. and D.G.) were tested as negative controls. Each subject was 30 informed by the investigators and signed informed consent before the test.

All patients showed a positive cutaneous reaction to the rParj1 allergen. PjB was capable of inducing Type I immediate hypersensitivity in 9 out of 10 of the tested 35 patients. PjA gave positive reaction in 3 out of 10 of the patients and the wheal areas induced by prick were reduced in size respect to that ones triggered by the

wild-type allergen. The PjC and PjD did not give any SPT reaction. None reactions have been observed when non allergic subjects were tested as reported in the following table II.

5 Table II: Skin prick test of the rPar J and its disulfide bond variants

Patient No.	RParj1	PjA	PjB	PjC	PjD
1	++++	-	-	-	-
2	+++	++	+++	-	-
3	++++	-	+++	-	-
4	++++	-	++++	-	-
5	++++	++	+++	-	-
6	++++	-	+++	-	-
7	++++	-	++++	-	-
8	++++	++	+++	-	-
9	+++	-	++	-	-
10	+++	-	++	-	-
P.C.	-	-	-	-	-
D.G.	-	-	-	-	-

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28

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CLAIMS

1. A variant of an allergen belonging to the family of the ns-LTP proteins, said variant lacking at least one of the four disulfide bridges constituting the structure of said allergen.
5
2. The variant according to claim 1, said variant lacking at least one disulfide bridge in the amino-terminal region of said allergen.
10
3. The variant according to claim 1 or 2, said variant lacking two of said disulfide bridges constituting the structure of said allergen.
4. The variant according to claim 1, said variant lacking three, or four of said disulfide bridges constituting the structure of said allergen.
15
5. The variant according to any one of the claims 1 to 4, said variant being a mutein wherein at least one of the cysteines constituting said disulfide bridges is deleted.
20
6. The variant according to any one of the claims 1 to 5, said variant being a mutein wherein at least one of the cysteines constituting said disulfide bridges is substituted with an amino acid residue not capable of forming disulfide bridges.
25
7. The variant according to claim 6, wherein said amino acid residue is serine or alanine.
8. The variant according to any of claims 1 to 7, wherein said variant has substantially the same length of said allergen.
30
9. The variant according to any one of the claims 1 to 8, wherein said allergen is one of the major allergens of Parietaria Judaica.
10. The variant according to claim 9, wherein said allergen is Parj1, and said cysteines constituting the disulfide bridges are the cysteines 4, 14, 29, 30, 50, 35 52, 75 and 91.

11. The variant according to claim 10, wherein the sequence of said variant comprises a sequence selected from the group consisting of the sequences reported in the sequence listing as SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 7.

5 12. The variant of anyone of claims 1 to 11 exhibiting reduced IgE binding capability while maintaining the capability of inducing IgG response.

10 13. The variant according to any one of the claims 1 to 12 for use as a medicament or as a diagnostic agent.

14. The variant according to claim 13 for use in a patient customised treatment and/or prevention of allergic form associated with an allergen belonging to the family of the ns-LTP proteins.

15 15. The variant according to claim 14 which is a variant of a major allergen of Parietaria Judaica and wherein the allergic form is associated with an allergen of Parietaria Judaica.

20 16. The variant according to claims 14 or 15 for use in the treatment of itch, erythema, edema, wheal, rash (urticaria) formation, rhino-conjunctivitis (seasonal allergies), bronchoconstriction, asthma and anaphylaxis.

17. The variant according to claims 13 to 16 for use in preventive specific immunotherapy.

25 18. The variant according to claim 13 for use as diagnostic agent for patient customised diagnosis of allergic forms.

19. A polynucleotide coding for the variant according to any one of the claims 1 to 12.

30 20. The polynucleotide according to claim 19, wherein said sequence of said variant comprises a sequence selected in the group comprising the sequences reported in the sequence listing as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8.

35 21. A vector comprising at least one polynucleotide according to claim 19 or 20.

22. An use of a variant according to any one of the

claims 1 to 12, for the preparation of a medicament for the treatment and/or the prevention of allergic form associated with an allergen belonging to the family of the ns-LTP proteins.

5 23. The use according to claim 22, wherein said allergen belonging to the family of ns-LTP proteins is the allergen corresponding to said variant.

10 24. The use according to claim 22 or 23, wherein said allergen is a major allergen of Parietaria Judaica and said allergic form associated with said allergen is an allergic form from Parietaria Judaica.

25. A pharmaceutical composition comprising at least one variant according to any one of the claims 1 to 12, and a pharmaceutically acceptable carrier.

15 26. A pharmaceutical composition comprising at least one polynucleotide according to claim 19 or 20, and/or one vector according to claim 21, and a pharmaceutically acceptable carrier.

20 27. A diagnostic agent comprising at least one variant according to any one of the claims 1 to 12, and a pharmaceutically acceptable carrier.

28. A kit for the derivation of a subject-customised allergogram for an allergic form associated with an ns-LTP allergen, comprising

25 - a first composition comprising said ns-LTP allergen in native form together with an acceptable carrier;

30 - at least one composition comprising a single variant of said ns-LTP allergen according to any one of the claims 1 to 12 and an acceptable carrier;

said allergogram being derivable contacting said compositions with immunoglobulins of said subject and observing the effects thus obtained.

35 29. The kit according to claim 28, comprising said first composition and a number of compositions each comprising a single variant of said ns-LTP allergen,

32

equal to the number of the variants of said ns-LTP allergen.

30. The kit according to claim 28 or 29, wherein said ns-LTP allergen is ParJ1.

	1	10	20	25	36	45
Parj	1	QETCGTMRV	AIMPCLLPFVQ	GKEKE	PSKGCCSGAKR	LDGETKTGP
Parj	2	EEACGGKVVQ	DIMPCLLHFVK	GEEKE	PSKECCSGTKK	QRVHACECLOT
		alpha 1	loop1	alpha 2	loop2	alpha 3
	56	62	71	88		
Parj	1	AMKTYS	DIDGKLVSE	VPKHCGIVVSKLPPIDV	NMDCK	TL
Parj	2	ATKGISGI	KNELVAEVP	KKCDIKTTLPPITA	DFDCS	KIQSTIFRGYY
	Loop3	alpha 4	loop4		beta	

Fig. 1

Fig. 2

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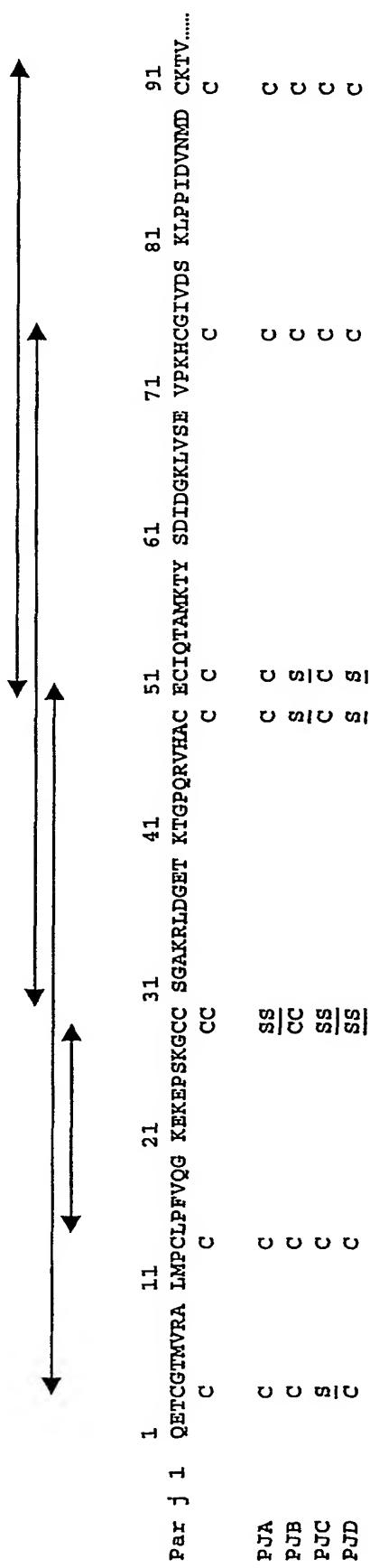


Fig. 3

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A**rParj1 PjA PjB PjC PjD****B****rParj1 PjA PjB PjC PjD****FIG. 4**

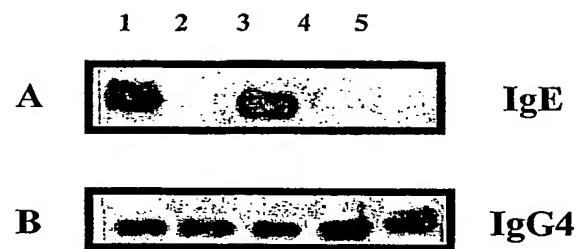


FIG. 5

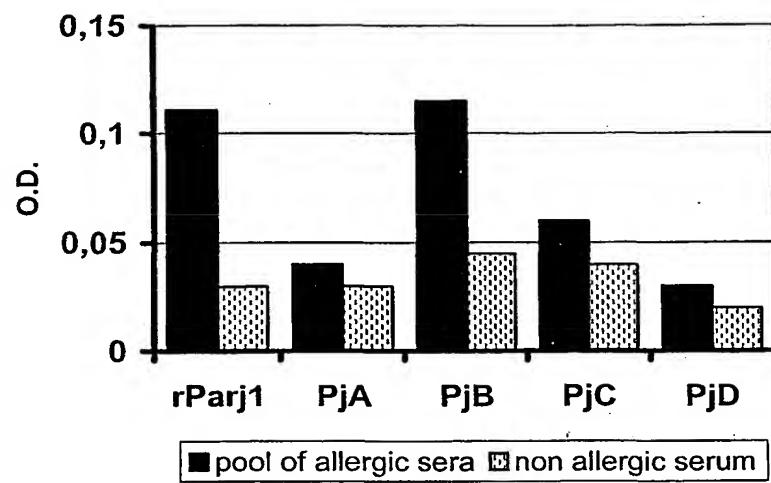


FIG. 6

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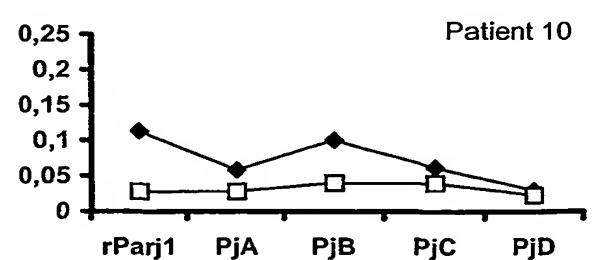
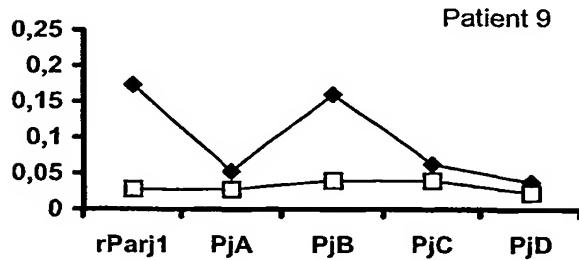
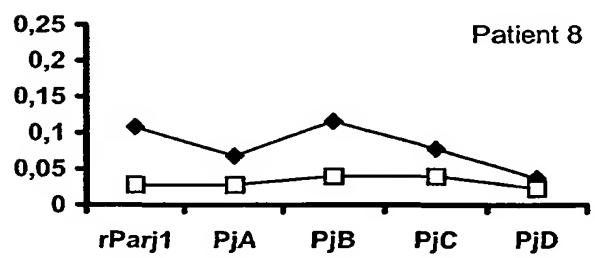
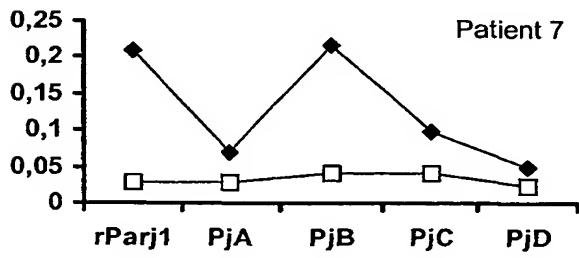
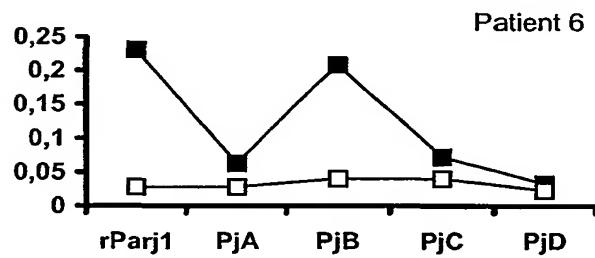
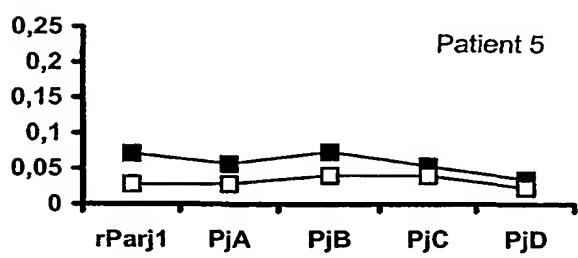
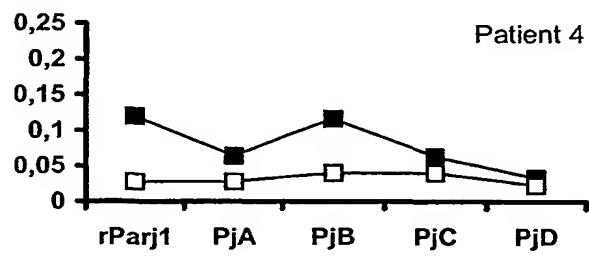
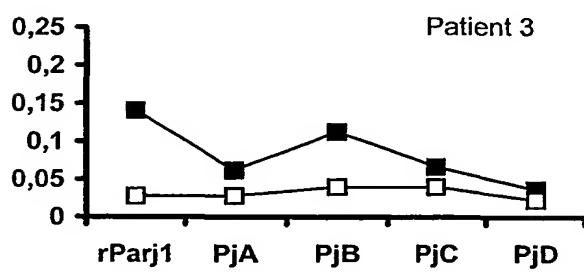
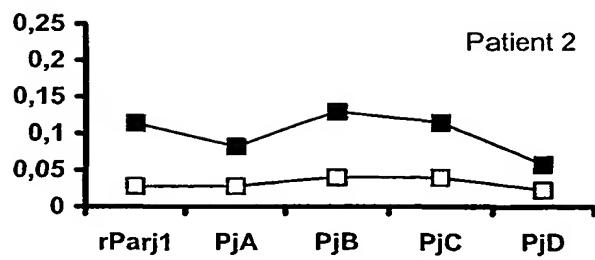
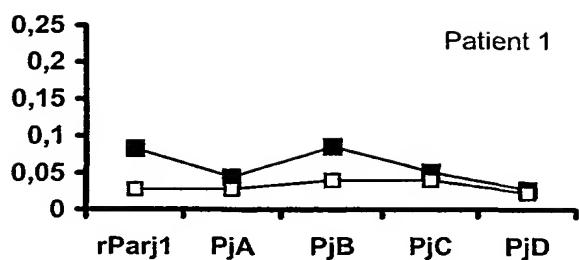


FIG. 7

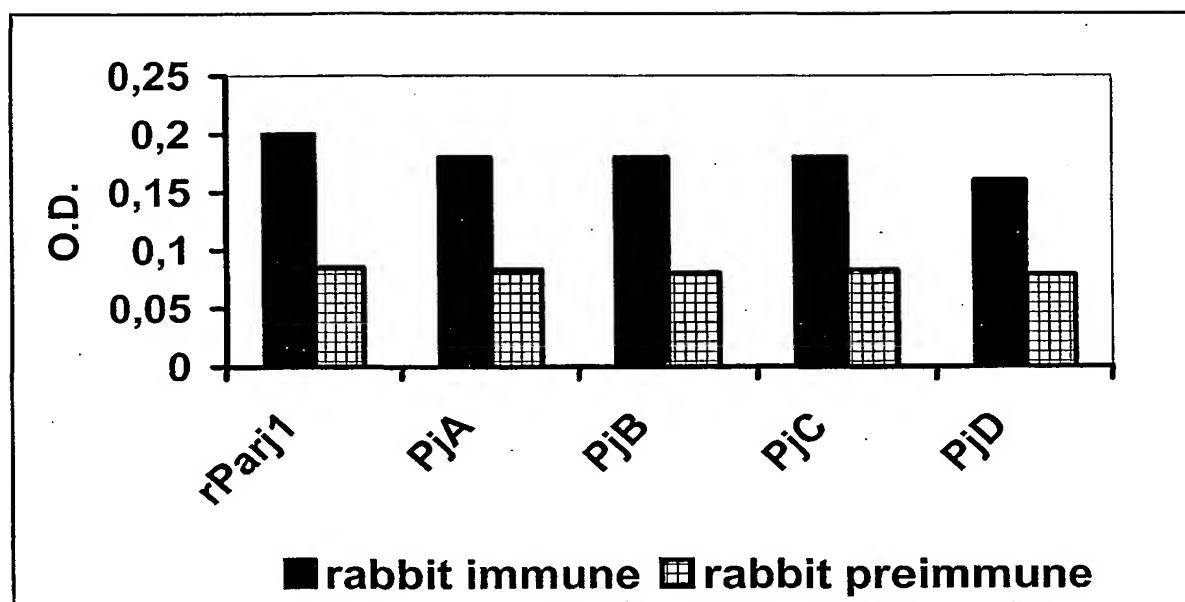


FIG. 8

SEQUENCE LISTING

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 96
Phe Val Gln Gly Lys Glu Lys Pro Ser Lys Gly Cys Cys Ser Gly
20 25 30

gcc aaa aga ttg gac ggg gag acg aag acg ggg ccg cag agg gtg cac
 144
Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His
35 40 45

gct tgt gag tgc atc cag acc gcc atg aag act tat tcc gac atc gac
 192
Ala Cys Glu Cys Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50 55 60
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35

40

45

Ala Cys Glu Cys Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50

55

60

Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65

70

75

80

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

85

90

95

Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

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105

110

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1 5 10 15

ttc gtg cag ggg aaa gag aaa gag ccg tca aag ggg agc agc agc ggc
96
Phe Val Gln Gly Lys Glu Lys Glu Pro Ser Lys Gly Ser Ser Ser Gly

20 25 30

gcc aaa aga ttg gac ggg gag acg aag acg ggg ccg cag agg gtg cac
144
Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His

35 40 45

gct tgt gag tgc atc cag acc gcc atg aag act tat tcc gac atc gac
192
Ala Cys Glu Cys Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50 55 60

ggg aaa ctc gtc agc gag gtc ccc aag cac tgc ggc atc gtt gac agc
240
Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65 70 75 80

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288

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

85

90

95

gtt cct cg^g caa ccc caa ctt cca gtc tct ctc cgt cat ggt ccc gtc
336

Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

100

105

110

acg ggc cca agt gat ccc gcc cac aaa gca cg^g ttg gag aga ccc cag
384

Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

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10

15

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20

25

30

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35

40

45

Ala Cys Glu Cys Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50

55

60

Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65

70

75

80

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

85

90

95

Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

100

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110

Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

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of Cys 50 and 52 with Ser

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48
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ttc gtg cag ggg aaa gag aaa gag ccg tca aag ggg tgc tgc agc ggc
96
Phe Val Gln Gly Lys Glu Lys Glu Pro Ser Lys Gly Cys Cys Ser Gly
20 25 30

gcc aaa aga ttg gac ggg gag acg aag acg ggg ccg cag agg gtg cac
144
Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His
35 40 45

gct agt gag agc atc cag acc gcc atg aag act tat tcc gac atc gac
192
Ala Ser Glu Ser Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp
50 55 60

ggg aaa ctc gtc agc gag gtc ccc aag cac tgc ggc atc gtt gac agc
240
Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser
65 70 75 80

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Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val
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ttc gtg cag ggg aaa gag aaa gag ccg tca aag ggg agc agc agc ggc
96
Phe Val Gln Gly Lys Glu Lys Glu Pro Ser Lys Gly Ser Ser Ser Gly

20

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30

gcc aaa aga ttg gac ggg gag acg aag acg ggg ccg cag agg gtg cac
144

Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His

35

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gct tgt gag tgc atc cag acc gcc atg aag act tat tcc gac atc gac
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gtt cct cgg caa ccc caa ctt cca gtc tct ctc cgt cat ggt ccc gtc
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Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

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Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

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50 55 60

Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65 70 75 80

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30, 50 and 52 with Ser

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Phe Val Gln Gly Lys Glu Lys Glu Pro Ser Lys Gly Ser Ser Ser Gly

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gcc aaa aga ttg gac ggg gag acg aag acg ggg ccg cag agg gtg cac
144

Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His

35

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45

gct agt gag agc atc cag acc gcc atg aag act tat tcc gac atc gac
192

Ala Ser Glu Ser Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50 55 60
ggg aaa ctc gtc agc gag gtc ccc aag cac tgc ggc atc gtt gac agc
240
Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser
65 70 75 80

aag ctc ccg ccc att gac gtc aac atg gac tgc aag aca gtt gga gtg
288
Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val
85 90 95

gtt cct cg^g caa ccc caa ctt cca gtc tct ctc cgt cat ggt ccc gtc
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Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val
100 105 110

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384
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Ala Ser Glu Ser Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

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Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65

70

75

80

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

85

90

95

Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

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Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

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INTERNATIONAL SEARCH REPORT

Int	nal Application No
PCT/IT 01/00471	

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 7	C12N15/28	C12N15/70	C07K14/415	A61K39/36	G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLOMBO P. ET AL: "Identification of an immunodominant IgE epitope of the <i>Parietaria judaica</i> major allergen" JOURNAL OF IMMUNOLOGY, vol. 160, no. 6, 15 March 1998 (1998-03-15), pages 2780-5, XP002186915 abstract	1,2,5-26
Y	see above page 2784; claims 1,2 ---	3,4, 27-30 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 January 2002	25/01/2002
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Celler, J

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/IT 01/00471

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FERREIRA F. ET AL: "Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy" FASEB J, vol. 12, February 1998 (1998-02), pages 231-242, XP002186916 page 232, column 1 page 237, column 1 -page 238, column 2 page 240, column 1 -column 2 ---	1-30
Y	OLSSON S. ET AL : "Contribution of disulphide bonds to antigenicity of Lep d 2, the major allergen of the dust mite Lepidoglyphus destructor" MOLECULAR IMMUNOLOGY, vol. 35, 1998, pages 1017-1023, XP001026605 abstract page 1021, column 2 -page 1022, column 1 ---	3,4, 27-30
A	WO 00 44781 A (UNIV CALIFORNIA) 3 August 2000 (2000-08-03) abstract page 1 -page 2 page 17 ---	1-30
P,X	BONURA A. ET AL: "Hypoallergenic variants of the Parietaria judaica makor allergen Par j 1: a member of the non-specific lipid transfer protein plant family" INT ARCH ALLERGY IMMUNOL, vol. 126, no. 1, September 2001 (2001-09), pages 32-40, XP001037388 the whole document -----	1-30

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 19-21(partly)

Present claims 19-21 relate to an extremely large number of possible polynucleotides. In fact, due to the degeneracy of the genetic code the claims contain so many possible permutations that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a complete search over the whole scope of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely polynucleotide sequences reported in the present sequence listing as Seq. ID. No.: 2, 4, 6 and 8.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IT 01/00471

Patent document cited in search report	Publication date		Patent family member(s)		Publication date
WO 0044781	A 03-08-2000		AU 2738300 A EP 1147131 A1 WO 0044781 A1		18-08-2000 24-10-2001 03-08-2000

Form PCT/ISA/210 (patent family annex) (July 1992)

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CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
14 March 2002 (14.03.2002)

PCT

(10) International Publication Number
WO 02/020790 A1

(51) International Patent Classification⁷: **C12N 15/28**,
15/70, C07K 14/415, A61K 39/36, G01N 33/68

(74) Agents: BRUNO, Enrica et al.; c/o Società Italiana
Brevetti S.p.A., Piazza di Pietra, 39, I-00186 Roma (IT).

(21) International Application Number: PCT/IT01/00471

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU,
ZA, ZW.

(22) International Filing Date:
11 September 2001 (11.09.2001)

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).

(25) Filing Language: English

Published:
— with international search report

(26) Publication Language: English

(48) Date of publication of this corrected version:
30 May 2003

(30) Priority Data:
RM2000A000494
11 September 2000 (11.09.2000) IT

(15) Information about Correction:
see PCT Gazette No. 22/2003 of 30 May 2003, Section II

(71) Applicant (*for all designated States except US*): CONSIGLIO NAZIONALE DELLE RICERCHE [IT/IT];
Piazzale Aldo Moro, 7, I-00185 Roma (IT).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): GERACI,
Domenico [IT/IT]; Piazza Boccaccio, 1, I-90144 Palermo
(IT). COLOMBO, Paolo [IT/IT]; Via Croce Rossa, 113,
I-90146. Palermo (IT). DURO, Giovanni [IT/IT]; Via
Principe di Paternò, 137, I-90145 Palermo (IT). IZZO,
Vincenzo [IT/IT]; Via Sacco e Vanzetti, 13, I-90121
Palermo (IT). COSTA, Maria, Assunta [IT/IT]; Via
Monti Iblei, 41, I-90144 Palermo (IT).

(54) Title: PARIETARIA JUDAICA NS-LTP ANTIGEN VARIANTS, USES THEREOF AND COMPOSITIONS COMPRISING THEM

WO 02/020790 A1

1 2 3 4 5

A



IgE

B



IgG4

(57) Abstract: The present invention relates to hypoallergenic variants of ns-LTPs allergens, to pharmaceutical compositions comprising them and to the use of such variants for the preparation of medicaments suitable in the treatment and in the prevention of the allergic forms associated with an ns-LTP allergen, in particular to the allergen corresponding to the variant used.

**PARIETARIA JUDAICA NS-LTP ANTIGEN VARIANTS, USES THEREOF AND
COMPOSITIONS COMPRISING THEM**

DESCRIPTION

Field of the invention

5 The present invention relates to the fields of the prevention and the treatment of allergic symptoms associated with allergens belonging to the non-specific Lipid Transfer Protein (ns-LTPs) family.

State of the art

10 Ns-LTPs proteins are small proteic molecules of approximately 10 KDa that demonstrate high stability, and are naturally present in all vegetal organisms studied to date. In several species they have also been identified as allergens, as in the case of the Rosaceae Prunoideae 15 (peach, apricot, plum) and Pomoideae (apple), and Graminaceae, as in the Urticaceae like Parietaria Judaica (18-23).

20 These proteins are characterized by their ability to transport lipids through membranes *in vitro*, an ability justifying their denomination and corresponding to at least some of the activities exerted *in vivo* (17).

25 However, in spite of the different functions and of the heterogeneity of their sequence, ns-LTPs have a highly conserved secondary structure, comprising four alpha-helices (separated by loops) and one folded beta layer arranged in the 5'-3' direction according to a α - α - α - β pattern.

30 This structure is provided by the presence of four disulfide bridges formed by eight cysteine residues present in the 4 alpha-helices in the fourth loop, in the folded beta-layer and in the amino-terminal region (cfr. ref. 29).

 In particular:

- a first disulfide bridge connects the amino-terminal region and the third alpha-helix,
- a second disulfide bridge connects the first alpha-helix and the third alpha-helix,

- a third disulfide bridge connects the second alpha-helix and the fourth loop, and

- a fourth disulfide bridge connects the third alpha-helix and the folded beta-layer.

5 These cysteine residues are highly conserved in all ns-LTPs, and with reference thereto a consensus sequence can be derived (17).

10 Despite their high conservation, given the sequence heterogeneity of ns-LTPs, no notation system for the residues forming disulfide bridges valid for all ns-LTPs exists, though those skilled in the art may easily single out such cysteines using the knowledge of the state of the art.

15 With particular reference to the Parj1 protein, and specifically to the mature ParJ1.0102 form, the cysteines apt to form disulfide bridges are the residues 4, 14, 29, 30, 50, 52, 75 and 91, and the related bridges are arranged in the order Cys4- Cys52 (first bridge), Cys14- Cys29 (second bridge), Cys30- Cys75 (third bridge), 20 Cys50- Cys91 (fourth bridge) (12).

25 Par J1, besides being an ns-LTP, represents, together with Par J2, one of the major allergens of the Parietaria Judaica (PJ), a plant whose pollen constitutes one of the most widespread environmental antigens, especially in the Mediterranean area (1).

In fact, Parietaria Judaica pollen contains at least nine allergens with molecular masses ranging from 10 to 80 KDa and different capabilities of binding IgE [1-9].

Thereamong, Parj1, in the two isoform par11.1.2 and 30 Parj1.0201 isolated from independent genes (10) and Parj2 acquire a remarkable relevance as major allergens. In particular these two ns-LTPs are capable of inhibiting the majority of specific IgE against Parietaria allergens, and, upon administration, both have an 35 immunological behaviour in all alike that of the commercial extracts commonly used (11).

However, ParJ1 and ParJ2 do not constitute the sole ns-LTPs having allergenic properties. Recently, some scientific papers describing the characterisation of new allergenic molecules homologous to the ns-LTPs have 5 been published (18-23).

Despite sequence heterogeneity, following cross-reactivity experiments between different ns-LTP allergens and related produced antibodies, it was demonstrated that ns-LTP constitute a widespread family of allergens (pan-allergen) as already described for profilin (19). 10

However, in comparison with the abundant information on the structure of this 'pan-allergen', exhaustive information on the localisation of the epitopes for IgE and IgG therein, as well as in the individual ns-LTP allergens (ParJ1 and ParJ2 included) are not available 15 (11, 12).

As a result of the mechanism in charge of the development of the allergic response, and of the verified role of IgG and IgE therein, the derivation of such a map would be instead of enormous relevance for the drafting 20 of a novel therapeutic approach to these allergic forms (13).

In particular, the derivation of molecules with reduced or even absent IgE binding capability, yet 25 concomitantly capable of inducing IgG response, and in particular of IgG4, might be a landmark both from a therapeutic and a preventive point of view.

Such a molecule would allow immunosuppression of the T cell response with reduced or even absent side effects.

30 In fact, to date the therapy of an undergoing allergy consists in the mere pharmacological cure of the allergic symptomatology.

A preventive therapy represented by the specific immunotherapy (SIT) actually consists in the subcutaneous 35 administration of diluted quantities of allergen to the patient so as to suppress the specific reaction towards the allergen.

The majority of the commercial protein extracts used therefor however, are anyhow crude extracts, mixtures of several components in which a precise standardization of the allergenic component is 5 difficult.

Thus, the SIT strategy can entail the administration of allergenic components towards which the patient is not sensitive, inducing the secretion of IgEs specific towards other components of the extract. Moreover, the 10 administration of the total allergen entails the possibility of side effects which, though with extremely low occurrence, could even cause anaphylactic shock.

Concerning in particular the Parietaria Judaica, epidemiological studies have also highlighted a different 15 distribution of the two major allergens in the human population (12 millions of affected subjects in the Mediterranean area) where, approximately 20% of the PJ allergic patients do not exhibit a concomitant presence of IgE specific against both allergens. Therefore, an 20 administration of total or partially purified crude extracts could entail an administration of major allergens to which the patient is not allergic.

Hence, the use of recombinant molecules, allowing a 25 patient customized diagnosis and therapy, could represent a valid alternative to the traditional use of crude extracts.

In particular, the characterization and the development of alternative molecules with reduced side 30 effects, i.e., having a reduced or absent interaction with the IgE while maintaining the capability of binding the IgGs (in particular the IgG4) with respect to the wild type and therefore the capability of immunosuppressing the T response, could allow to implement an alternative approach overcoming the 35 disadvantages inherent to the traditional approach.

Such an alternative molecule with reduced anaphylactic capacity were in fact sought by producing

crude formaldehyde- or glutaraldehyde- polymerised extracts (16).

Although effective, as demonstrated by clinical trials, these modified molecules have proved however to 5 present the abovedescribed disadvantage of a difficult standardization of the extracts.

Following the advent of genetic engineering both recombinant allergens immunologically similar to the native allergens (14 and 15), and recombinant allergen 10 having instead a reduced allergenic activity with respect to the allergen wild type (therefore therapeutically suitable as a substitute of the latter), have been derived in a pure form.

None of such a mutant have not however been derived 15 with particular reference to the ns-LTPs allergens.

SUMMARY OF THE INVENTION

An object of the present invention is a variant of 20 an allergen belonging to the ns-LTP protein family, specifically a hypoallergenic variant of a complete allergen or fragment thereof of the ns-LTD protein family.

In particular object of the invention is a variant 25 of an allergen belonging to the ns-LTP family which lacks at least one of the four disulfide bridges constituting the structure of said allergen.

A first advantage of the variant of the present invention is that with respect to the native allergen it has a reduced or even absent capability of binding IgE, having concomitantly an intact capability of binding IgG 30 of the said subjects.

This differential binding capability is particularly enhanced in the variants wherein such a missing bridge be localized in the amino-terminal region of the allergen at the domain alpha-helix 1- loop 1- alpha-helix 2, as they 35 have a particularly reduced IgE binding activity, especially in the variants lacking at least two disulfide bridges.

In case such a variant be lacking three, or all four, of the disulfide bridges of the native allergen, the relevant IgE binding activity is reduced up to be substantially absent. Such a variant constitutes 5 accordingly a preferred embodiment of the invention.

The relevance of such a differential binding capability of the variant of the invention lies in that according to the role of the two immunoglobulins in the molecular mechanism of the allergic response evidenced in 10 the above paragraph, it turns out in molecules, that although immunogenic have a reduced or absent allergenicity.

Variants of the invention which mainly maintain most of amino acid sequence of the wild type allergen, and has 15 accordingly substantially the same length of the said allergen, constitute in this connection a preferred embodiment of the invention.

In particular variants consisting of a mutein in which at least one of the cysteines constituting said 20 disulfide bridges is deleted, or substituted with an amino acid residue not capable of forming disulfide bridges, are preferred.

In this latter case, the substitution of the cysteine residue with serine or alanine, as amino acids 25 tested compatible with the ns-LTP α - α - α - α - β structure, proved particularly effective.

The embodiment related to variants of the major allergens of Parietaria Judaica is particularly preferred.

30 In particular, the Parj1 variants, specifically the Parj1 muteins in which the deleted or substituted residues are the cysteines 4, 14, 29, 30, 50, 52, 75 and 91, and in particular the variants having a sequence selected in the group comprising the sequences reported 35 in the sequence listing as SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 10, are especially relevant.

Object of the present invention is also a polynucleotide coding for the variants of the present invention, in particular for the above indicated muteins, and specifically the polynucleotides comprising a sequence selected in the group comprising the sequences reported in the sequence listing as SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7 and SEQ ID NO: 9, as well as the vectors comprising them.

In the light of what set forth above, object of the present invention are also any of the above mentioned variants for use as medicament or as a diagnostic agent, and in particular for use in the treatment and/or the prevention and/or the diagnosis of the allergic form associated with an allergen belonging to the family of ns-LTP proteins, (this in light of the pan-allergen characteristic verified for the ns-LTP allergens), in particular to the allergen corresponding to said variant.

In the specific case, hereto disclosed by way of example, of major allergens of Parietaria Judaica showing an uneven ability to stimulate serum IgE production in allergic patients, a specific diagnosis for the individual allergen may be attained using the variants of the invention, e.g., as follows: initially the recombinant version of each native molecule (parJ1 and Parj2) is used for a specific diagnosis of the allergy by skin prick test. Patients sensitive to one of two allergens can then be analyzed for positiveness to the individual allergen variants to which they tested positive in order to highlight negatively testing variants. Then, such variants can be administered in substitution of commercial protein extracts developing an allergen-specific immunotherapy. Further modes of employ for diagnostic as well as therapeutic ends are anyhow derivable by those skilled in the art in light of the knowledge of the state of the art.

Object of the present invention is also a pharmaceutical composition comprising a therapeutically

effective quantity of at least one of the variants, or a polynucleotide or a vector among the above mentioned ones, and a pharmaceutically acceptable carrier, as well as all the matter compositions comprising at least one of the above mentioned molecules and one carrier chemically compatible therewith.

This pharmaceutically and/or chemically acceptable carrier can be any one carrier known to the art as suitable in pharmaceutical or matter compositions containing the molecules like the above mentioned ones, therefore in particular peptides and conjugates and/or oligonucleotides in any form, in particular in solid and in liquid form; an example of composition in liquid form is provided by compositions whose carrier is water, saline solutions, like, e.g., solutions containing NaCl and/or fosfate, or other solutions containing buffer molecules.

A still further object of the present invention is a kit for the derivation of a subject-customised allergogram, for an allergic form associated with an ns-LTP allergen comprising

- a first composition comprising said ns-LTP allergen in native form together with a chemically and/or pharmaceutically acceptable carrier;
- at least one composition comprising a single variant of said ns-LTP allergen as abovedescribed and a chemically and/or pharmaceutically acceptable carrier;

said allergogram being derivable contacting said compositions with immunoglobulins of said subject and observing the effects thus obtained.

Particularly preferred are the embodiments in which the kit comprises, besides said first composition, a number of compositions each comprising a single variant of said ns-LTP allergen, equal to the number of variants of said ns-LTPs allergen and that in which said allergen is a Parietaria Judaica allergen, specifically ParJ1 (in any one form thereof) or ParJ2 (in any one form thereof).

In particular such compositions can be contacted with immunoglobulins by 'skin prick test' *in vivo*, or on patient's tissues like, e.g., blood, *in vitro*. Other modes of employ of the kit of the present invention to diagnostic ends are derivable by those skilled in the art in light of the knowledge of the state of the art. The invention will be better described with the aid of the attached figures.

DESCRIPTION OF THE FIGURES

Fig. 1 reports the amino acid sequences of the native Par J 1.0102 and Par j 2.0101 aligned therebetween and with respect to the three-dimensional structure thereof. The notation of the amino acids relates to the sequence of the Par j 1.0102.

Fig. 2 shows the amino acid sequences of the Par j 1.0101 and of some ns-LTP proteins aligned thereamong. The arrows indicate the disulfide bridges present in the three-dimensional structure of the proteins. The amino acids are indicated in one-letter code. The Cs reported in the last row of the table indicate the cysteine residues conserved in all proteins of the ns-LTP family.

Fig. 3 reports the schematic representation of the mutants of the major allergen of the Parietaria Judaica Par j 1.0102, the sequence thereof being reported in the sequence listing as SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 10. The amino acids are reported in one-letter code. The underlined amino acids indicate the mutations effected in the native sequence. The arrows indicate the disulfide bridges.

Fig. 4 reports in panel A the Western blot analysis results showing the IgE binding activity of the rParj1 and its disulfide bond variants by using a pool of sera (n=30) from monosensitive Pj allergic patients.

Panel B shows a Coomassie Brilliant Blue staining of the recombinant proteins used.

In both panels on the first lane the result referred to the native Par j 1.0101 is reported; on the second

lane the result referred to the mutant PjA is reported; on the third lane the result referred to the mutant PjB is reported; on the fourth lane the result referred to the mutant PjC is reported; and on the fifth lane the result referred to the mutant PjD is reported.

Fig. 5 shows in panel A the outcome of a Western Blot analysis on a pool of sera from PJ allergic patients, aimed at demonstrating the IgE binding capability (activity) of some mutants of the present invention extensively disclosed in example 3.

On the first lane the result referred to the native Par j 1.0101 is reported; on the second lane the result referred to the mutant PjA is reported; on the third lane the result referred to the mutant PjB is reported; on the fourth lane the result referred to the mutant PjC is reported; and on the fifth lane the result referred to the mutant PjD is reported.

In panel B the outcome of a Western blot analysis on a pool of sera from PJ allergic patients, aimed at demonstrating the IgG4 binding capability of the same abovedescribed mutants, it also extensively described in example 3, is shown.

On the first lane the result referred to the native Par j 1.0101 is reported; on the second lane the result referred to the mutant PjA is reported; on the third lane the result referred to the mutant PjB is reported; on the fourth lane the result referred to the mutant PjC is reported; and on the fifth lane the result referred to the mutant PjD is reported.

Fig. 6 reports an histogram showing the results of the ELISA detection experiment carried out using the pool of sera used in example 3, extensively described on the example 4. Black histogram indicate results obtained with allergic sera; dotted square indicated results obtained with non allergic serum. On the y axis the optical density measured and in the x axis the proteins tested (the rParj1 and its disulfide bond variants PjA, PjB,

PjC, and PjD)) are reported.

Figure 7 shows ten diagram reporting the results of the ELISA detection carried out using monosensitive sera from ten Pj allergic patients extensively described on example 4, each diagram corresponding to a respective patient.

On each diagram on the y axis the optical density measured and in the x axis the proteins used (the rParj1 and its disulfide bond variants PjA, PjB, PjC, and PjD)) are reported.

On each diagram black squares indicate allergic sera, white squares a non allergic serum.

Fig. 8 reports an histogram showing the results of the ELISA detection of the Ig binding activity of a rabbit polyclonal immune and pre-immune antisera against rParj1, extensively described on the example 6.

On the y axis the optical density measured and in the x axis the antigens used (the rParj1 and its disulfide bond variants PjA, PjB, PjC, and PjD)) are reported.

Black squares indicate results obtained on rabbit immune, checkered squares results obtained on rabbit preimmune.

DETAILED DESCRIPTION OF THE INVENTION

The experimental approach that led to the present invention consisted of a mutagenesis strategy aimed at the targeted disruption of all the disulfide bridges present in all the ns-LTPs. This in order to generate molecules with reduced or absent affinity to IgE, yet with intact affinity to the other classes of antibodies apt to compete with the specific IgEs against the native allergen. The ns-LTPs allergen ParJ1, whose sequence and structure is reported in comparison with Par J2 in figure 1, and in particular the isoform Par j 1.0102 (the primary sequence thereof being reported in the annexed sequence listing as SEQ ID NOS:1 and 2), was taken as a molecular model, to be used for carrying out mutagenesis

and the subsequent verification of the properties of the obtained mutant. In particular, recombinant DNA technology was resorted to in a strategy of site-directed mutagenesis against cysteines 4, 29, 30, 5 50 and 52, i.e., the cysteines constituting the disulfide bridges according to the structural model known to the art.

The results of these experiments demonstrate the close relationship existing between the three-dimensional structure of the protein and the capability of forming epitopes for the IgEs, and in particular that the gradual disruption of the disulfide bridges causes a reduction of the serum IgEs binding activity thereof, whereas it does not affect the IgG4 binding activity thereof.

This in light of the Western blot analysis described in example 3, and in figure 5, in which lanes 2, 3 and 4 (see Fig. 5 panel A) show the reduction of the serum IgEs binding activity by the mutants of the present invention, which should be construed as three-dimensional mutants.

In particular, the Cys29-Cys30 mutant (PjA) shows a very weak binding band (Fig. 5 lane 2) whereas the Cys50-Cys52 mutant (PjB) is somehow still capable of binding the IgEs (Fig. 5, lane 3). Instead, more remarkable is the result shown by the PjC and PjD mutants (Fig. 5, lanes 4 and 5) for which no binding to human IgEs can be highlighted.

Such results have been confirmed by ELISA and IgE inhibition assays (see examples 4 and 5) where the PjB was the only variant still able to bind Parj1-specific IgE antibodies in solution while the other variants exhibited a very low inhibition capacity. The loss of additional disulfide bridges (PjC and PjD) leads to the absence of any IgE recognition (see Example 4 and Figure 4).

These results all together show that ns-LTP Parj1 variants lacking of at least one disulfide bridge have a reduced allergenicity, which is even absent in the

variants wherein the lacking bridge is localized in the aminoterminal region of the allergen at the domain alpha-helix1-loop1-alphahelix2, in particular when the lacking bridges are at least two.

5 The maintenance by these variants of an overall antigenicity that, notwithstanding the reduced or absent allergenicity, is comparable or identical to the one of the wild type allergen, has been shown by experiments
10 wherein binding activity of wild-type allergen and its variants to antibodies different than IgE, have been compared.

Such experiments are Western Blots carried out using
as antibodies rabbit polyclonal antibodies and IgG4 of Pj
allergic patients, extensively described in examples 6
15 and 3 respectively.

In these experiments the hypoallergenic variants generated by genetic engineering presented a similar behavior compared to the wild type, with a low reduction
20 of their binding activity towards the anti-rParj1 rabbit antibodies.

Accordingly, a variant that lacks of at least one disulfide bridges still contains several protein domains similar to the native molecule and, although at different extent, is apt to induce the production of IgG antibodies
25 (see example 3 and 6). IgE production and/or IgE-mediated presentation of the allergen, would be prevented by such "blocking" antibodies and reducing T cell proliferation and release of cytokines (25).

The above data have been confirmed also *in vivo* in particular by Skin Prick Test (SPT) analysis as described in example 7, where the pure recombinant proteins were tested on ten PJ allergic patients (the same analyzed by ELISA in example 4 and figure 7).

With regard to the single mutants, PjA showed a very low IgE binding activity and only 3 out of 10 patients with cutaneous Type I hypersensitivity and a reduced wheal area respect to that one induced by wild-type

allergen. On the contrary, loss of the Cys50-Cys91 and Cys4-Cys52 bridges seems to have a minor effect since an IgE binding activity and a positive SPT are still present. The loss of additional disulfide bridges (PjC and PjD) leads to the absence of any cutaneous reaction (see example 7).

These results obtained analyzing individuals demonstrate the reliability of the above described data *in vitro*, and above all that while the disruption of the disulfide bridges in the amino terminal region (in the specific case Cys4-Cys52 and Cys50-Cys91) affect even if not markedly the human IgE binding capability of this mutant, the disruption of all the four bridges (in the specific case Cys4-Cys52, Cys14-Cys29, Cys30-Cys75 and Cys50-Cys91) have a devastating effect on the IgE recognition, and therefore on the allergenic response.

Accordingly, concerning the development of therapeutically useful hypoallergenic molecules, variants lacking of three or four bridges, as in the specific case PjC and PjD mutants, are considered as preferred embodiments. The above was demonstrated using a pool of sera as well as a cohort of individual patients, indicating that the obtained result is representative of the immune response of the allergic population.

It is pointed out that although obtained using specific variants derived by mutating the wild type allergen, these results are in fact not limited to the said specific variants, neither to the techniques used for the relevant derivation.

As such results are consequent to the modification of the three-dimensional structure of the allergen, they could anyhow have been obtained by any mutagenesis allowing the disruption of the disulfide bridges.

Accordingly any variant obtainable by deletion, substitution and/or the insertion of one or more amino acidic residue which results in variants lacking of at

least one disulfide bridge is included in the object of the invention.

The strategy of point mutation has however the remarkable advantage of allowing the insertion of minimal variations at the level of the primary sequence of the native protein and therefore of generating mutants that are more likely not to interfere with the variant recognition operated by the T cells, and above all the possibility to generate proteins having a high reproducibility.

Variants having substantially the same length of the wild type are accordingly considered preferred.

With regard to the techniques used for deriving the variants of the invention, it is not limited to the genetic engineering ones, as they are obtainable by techniques like chemical mutagenesis (formaldhehyde and gluteraldhehyde) which allow the disruption of disulfide bridges even in absence of mutations.

The genetic mutagenesis imply however the remarkable advantage of allowing the generation of proteins having a high reproducibility, while the, chemical mutants do not ensure a denaturation pattern constant at every preparation.

Furthermore, as the strategy described herein is independent on the epitope sequence on itself since it is based on the modification of the three-dimensional structure of the IgE determinants, the adopted mutagenesis strategy is actually independent from the primary sequence of the allergen (and therefore from the sequence of the specific IgE epitopes).

For this reason variants of all the proteins with allergenic activity belonging to the ns-LTP family (including Parj2), are included in the object of the invention due to the conserved structure (cfr. e. g.s figure 2 wherein Par j1 sequence is reported in comparison with the sequence of other ns-LTPs together with the placement of the disulfides bridges).

In particular variant of the invention is not only any other mutein of the ParJ1 allergen or of other ns-LTP allergens which, independently from the mutation carried out (substitution and/or deletion of one or more amino acid residues) and of the way in which such a mutation is carried out (e.g., by the above mentioned techniques), retain a structure equivalent to that of the corresponding native allergen lacking at least one disulfide bridge.

Thus the disruption of the disulfide bridges in ns-LTP allergens underlies *per se* a limited or absent IgE binding ability of patients allergic to the related variants.

Moreover, in particular in light to what is known in the art concerning the high conservation of the structure and the cross-reactivity that have led to the singling out of the so-called ns-LTP pan-allergen (see above) these data are indicative not merely of a suitability in the therapy and prevention of the allergic forms caused by the allergens corresponding to the individual variants, but also in the therapy and prevention of allergic forms caused by ns-LTP allergens other than those corresponding to the variants used.

A person skilled in the art can derive on the basis of his knowledge any information suitable for deriving uses, compositions and kit described in the summary of the invention.

With the aid of the following examples, a more detailed description of specific embodiments will now be given, in order to give a better understanding of the objects, characteristics, advantages and operating methods of the present invention.

EXAMPLES

Example 1: Cloning and Expression of Par J 1.0102

For the production of the major allergen of Parietaria Judaica Par j 1.0102 the pQE30 prokaryotic vector (Qiagen) was used. The latter characteristically

expresses recombinant proteins fused to a short histidine tail and inducible with isopropyl- β -D-thiogalactoside (IPTG). The histidine residues allow the purification of the recombinant protein by affinity chromatography.

For this reason, 1 ng of the P5 clone containing the processed version of the Par j 1.0102 (EMBL accession number X77414), the sequence thereof being reported in the annexed sequence listing as SEQ ID NO: 12, was subjected to 30 cycles of polymerase chain reaction (PCR) amplification at the following design: 94°C for 1 min, 52°C for 1 min, 72°C for 1 min. The synthetic primer oligonucleotides P5 forward and P5 reverse, the sequence thereof being reported in the annexed sequence listing as SEQ ID NO:11 and SEQ ID NO: 12, respectively, were used.

The fragment thus generated was fractionated on 1% agarose gel in 1 X TBE, extracted, purified and digested with Bam H1 and Hind III restriction enzymes and cloned in the pQE30 VECTOR (Qiagen) previously digested with the same enzyme. The linearized vector and the digested fragments were incubated for 4 hours at 16° C in presence of the enzyme DNA ligase according to different stoichiometric ratios. The reaction mixture was then transformed in the bacterial strain M15. The recombinant clones were sequenced with the method of Sanger and the nucleotide sequence thus determined demonstrated that the DNA fragment inserted into the pQE30 vector was identical to that known in the art (10).

Example 2: Cloning and expression of conformational mutants of ParJ 1.0102

PjA mutant (Cys29→Ser and Cys30→Ser) was generated using the Transformer Site-Directed Mutagenesis kit (Clontech) following the manufacturer's instruction and using the oligonucleotide P5 (29,30) reported in the sequence listing as SEQ ID NO: 13 (mapping from nucleotide 88 to nucleotide 105) and the Parj1 sequence as a template. PjB mutant (Cys50→Ser and Cys52→Ser) was

generated by PCR using as primers the oligonucleotide P5 (50-52) reported in the sequence listing as SEQ ID NO: 14 (mapping from nucleotide 91 to nucleotide 165) and P5 reverse oligonucleotide and 1 ng of the Parj1 clone as a template. The PCR fragment was digested with Pst I and Hind III restriction enzymes and ligated with the Pst I-Hind III linearized plasmid vector containing the Parj1 sequence (expressing the first 31 amino acids of the wild type Par j 1.0102 allergen). PjC mutant (Cys4→Ser, Cys29→Ser and Cys30→Ser) was generated by PCR amplification using the PjA variant as a template. The cysteine residue at position 4 was mutated by PCR using the oligonucleotides P5(triple), the sequence thereof being reported as SEQ ID NO: 15, and P5 reverse.

After purification, PCR fragment was digested with Bam HI and Hind III enzymes and cloned in the pQE30 vector previously digested with the same restriction enzymes. PjD mutant (Cys29→Ser, Cys30→Ser, Cys50→Ser and Cys52→Ser) was generated using the Transformer Site-Directed Mutagenesis kit (Clontech) following the manufacturer's instruction and using the synthetic oligonucleotide P5 (29,30) reported in the sequence listing as SEQ ID NO: 13 and the PjB variant as a template. All clones were sequenced with the method of Sanger (24) and the mutations and the open reading frames confirmed (See Fig.3 for details).

With this process 4 independent mutants, hereinafter designated PjA (SEQ ID NO: 3 and SEQ ID NO: 4); PjB (SEQ ID NO: 5 and SEQ ID NO: 6), PjC (SEQ ID NO: 7 and SEQ ID NO: 8), and PjD (SEQ ID NO: 9 and SEQ ID NO: 10) were isolated.

Example 3: Purification of recombinant proteins evaluation of the relevant capability of binding IgE of allergic patients

10 ml O/N culture of the recombinant clones (NM15 strain, Quiagen) were then used for an inoculation in 400 ml of 2YT broth (Bacto-tryptone 16 gr/l, Bacto-yeast 10

gr/l, NaCl 5 gr/l, pH 7,0) containing ampicillin and kanamycin at a final concentration of 100 µgr/ml and 10 µgr/ml, respectively.

A 1:40 dilution was grown for 1 hour at 37°C and, 5 after that, induced with 1 mM isopropylthio- β -galactoside for 4 hours at 37° C. Cells were harvested by centrifugation and the recombinant proteins purified by using the His Trap kit (Pharmacia) following the manufacturer's instructions. Recombinant proteins, 10 binding the HiTrap chelating column, were eluted using a buffer containing: 20mM phosphate buffer pH7.4. 0,5 M NaCl, 8 M UREA and 500 mM imidazole; fractions were analysed by 16% SDS-PAGE and Coomassie Brilliant Blue staining. Fractions containing the purified protein were 15 then diluted 1:100 in a buffer containing 20mM phosphate buffer pH7.4. 0,5 M NaCl and 20 mM imidazole to allow refolding of the protein, reloaded on the His Trap column and eluted with a buffer with no denaturing agents (20mM phosphate buffer pH7.4. 0,5 M NaCl and 500 mM imidazole). 20 Recombinant proteins were then desalted using a centrifugal filter device (Centriprep, Millipore) and analysed for their capability of binding human IgE from Pj allergic patients by Western blot as previously described (12), using a pool of sera (n=30) of Pj 25 allergic patients which did not receive any specific immunotherapy.

This analysis showed that the PjB mutant was still capable of binding human IgE while the PjA mutant retains only a weak IgE binding activity. The PjC and PjD mutants did not show any IgE binding activity suggesting that the IgE recognition was dependent on the three-dimensional folding of the protein (Fig.4 Panel A).

After that, membranes were stripped and reprobed 30 with a His-tag specific reagent (INDIA™ Hisprobe-HRP, Pierce, USA) to check that the IgE-allergen complex was 35 specific for the recombinant fused proteins. The concentration of the recombinant proteins was determined

by densitometric analysis of SDS-PAGE gels stained with Coomassie Brilliant Blue (see figure 4 panel B).

As a confirmation of this experiment another Western blot carried out using IgE and IgG4 of allergic patients.

Then, the proteins purified were fractionated on 16% PAGE-SDS and transferred on nitro-cellulose thanks to a Dry-blot system (Millipore). The membrane was incubated for 12-14 hours with a pool of sera from Pj allergic patients (1:5 dilution) in PBS-tween. The protein-human IgE and IgG4 binding complexes are highlighted using respectively a secondary anti-IgE and anti-IgG4 antibodies conjugated to radish peroxidase. Thus, the complexes are highlighted using a chemiluminescence system (Super-signal, Pierce). The relevant results are reported in figure 4.

Example 4: Elisa detection

The same pool of allergic sera from non-sensitive PJ allergic patients used in example 3 has been used in an ELISA experiment, showing the IgE binding activity of the rParj1 and its disulfide bond variants. A non allergic subject has been tested as a negative control on the ELISA.

The results confirm the pattern of reaction of the experiment of the example 3 (Fig.4 Panel A) with the PjB variant reacting in a way comparable to the wild-type allergen. A non allergic serum is shown as a negative control (Fig.6).

The IgE binding activity of the four Parj1 disulfide bond variants was also tested by ELISA using sera from ten monosensitive Pj allergic patients. Analysis of single sera showed a remarkable homogeneity of the reaction. In particular, the Cys4-Cys52 and Cys50-Cys91 bridges did not influence the allergenicity of the protein since this mutant (PjB) showed an IgE binding activity comparable to the wild-type allergen. On the other hand, the Cys14-Cys29 and Cys30-Cys75 bridges seem to be crucial for the IgE recognition. All the variants

lacking those two bonds (PjA, PjC and PjD) presented low or even absent IgE binding activity. (Fig. 7)

ELISA detection has been performed by adding 200 µl of a solution containing 5 µg/ml of antigen in coating buffer (sodium carbonate buffer pH 9,5) to each well of polystyrene plates overnight at room temperature. After several washing steps (1XPBS, 0,1% Tween 20) plates were saturated with a solution containing 5% BSA, 0,5% Tween 20 in coating buffer. After washing, 200 µl of serum (1:5 dilution) from Pj allergic patients or from a non allergic subject were incubated for 4 hours at room temperature. Bound IgE antibodies were detected with a goat antihuman IgE-HRP conjugate (Biosource International) diluted at a concentration of 0,5 ng/ml in 1XPBS, 0,25% BSA, 0,1% Tween 20 for 1 hour at room temperature. After several washes, colorimetric reaction was developed by adding 0,2 ml/well of substrate solution (0,4 mg/ml o-phenylenediamine in 0,1 M citrate buffer). Optical density was read at 495 nm in a BIO-RAD microplate reader.

Example 5: IgE inhibition assay

In order to investigate whether the disulfide bond variants were able to inhibit the binding of the IgE to the rParj1, increasing amount of each recombinant mutant were incubated with a pool of sera (n=10) of Pj monosensitive allergic patients.

The ability of the Parj1 disulfide variants to interact with IgE antibodies was determined by an ELISA inhibition experiment. A pool of sera (1:5 dilution) from ten monosensitive Pj allergic patients was preincubated overnight with increasing concentration of each disulfide bond variant (0,25-20 µg/ml of protein). The solutions were added to the ELISA wells coated with 5mg/ml of rParj1 and the ELISA steps were performed as above described. Percentage of inhibition was calculated according to the formula: % = 100-OD_A/OD_BX100, where OD_A

and OD_B represent the optical density read with the inhibited and non-inhibited pool of sera respectively.

5 The results are reported in the following Table I
Table I: Inhibition of IgE binding

Protein tested	%inhibition
rParj1	95%
PjA	16%
PjB	85%
PjC	14%
PjD	15%

10 The data reported in Table I suggests that all the variants lacking, at least, Cys14-Cys29 and Cys30-Cys75 disulfide bonds exhibit a comparable low level of inhibition (about 15%). On the contrary, the PjB variant (Cys50→Ser and Cys52→Ser) showed a high percentage of inhibition retaining a substantial ability of binding human IgE (about 85%).

15 Example 6: Rabbit polyclonal binding activity

Rabbits were immunized by PRIMM srl (Milan, Italy) using the rParj1 allergen. As a control, rabbit polyclonal antibodies were analysed on a Western blot using a *Parietaria judaica* crude extract detecting a band 20 of about 14000 Da corresponding to Parj1 native molecular weight. ELISA plates were coated at the same conditions as above described, with the wild-type Parj1 and with equal amount of each recombinant disulfide bond variant, were probed with an anti-rParj1 specific polyclonal serum 25 to analyse their binding activity.

Rabbit preimmune and immune sera were diluted at a concentration of 6 ng/ml and 200 µl of these solutions were incubated at room temperature for 1 hour. Wells were washed three times in 1XPBS, 0,1% Tween 20 and bound 30 antibodies were detected using a donkey antirabbit Ig HRP

linked (Amersham) at a 1:1000 dilution. Colorimetric reaction and optical density were performed as above described.

The data obtained suggest that the PjA, PjB and PjC variants show a similar behavior exhibiting a slight reduction of their binding ability (about 10%) compared to the rParj1 binding. The PjD variant showed a reduced binding activity (about 20%) while the preimmune serum did not show any reactivity towards the proteins (Fig.8).

10 Example 7: Skin Prick test experiments with purified miteins

Ten patients, with a clear history of *Parietaria judaica* allergy and with skin prick test (SPT) monosensitivity to Pj commercial extract, were analysed 15 in this study. All the patients did not receive immunotherapy against Pj pollen and were not receiving glucocorticosteroid treatment. Allergens were used at 1 μ g/ml concentration diluted in 0,9% NaCl. About 20 μ l of the test solution was placed on the forearms at a 20 distance of more than 2.5 cm between each prick. All tests were performed in duplicate. Histamine was used as positive control and 0,9% NaCl solution as a negative control. Reactions were measured after 20 min. By comparison with the wheal area generated by histamine 25 (100%), positive SPT were divided in three classes: 4+ were assigned to SPT with an area \geq 100% of area induced by histamine; 3+ were assigned to an area \geq 80-100% and 2+ to an area \geq 50-80%. Two non-allergic patients (P.C. and D.G.) were tested as negative controls. Each subject was 30 informed by the investigators and signed informed consent before the test.

All patients showed a positive cutaneous reaction to the rParj1 allergen. PjB was capable of inducing Type I immediate hypersensitivity in 9 out of 10 of the tested 35 patients. PjA gave positive reaction in 3 out of 10 of the patients and the wheal areas induced by prick were reduced in size respect to that ones triggered by the

wild-type allergen. The PjC and PjD did not give any SPT reaction. None reactions have been observed when non allergic subjects were tested as reported in the following table II.

5 Table II: Skin prick test of the rPar J and its disulfide bond variants

Patient No.	RParj1	PjA	PjB	PjC	PjD
1	++++	-	-	-	-
2	+++	++	+++	-	-
3	++++	-	+++	-	-
4	++++	-	++++	-	-
5	++++	++	+++	-	-
6	++++	-	+++	-	-
7	++++	-	++++	-	-
8	++++	++	+++	-	-
9	+++	-	++	-	-
10	+++	-	++	-	-
P.C.	-	-	-	-	-
D.G.	-	-	-	-	-

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28

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CLAIMS

1. A variant of an allergen belonging to the family of the ns-LTP proteins, said variant lacking at least one of the four disulfide bridges constituting the structure of said allergen.
5
2. The variant according to claim 1, said variant lacking at least one disulfide bridge in the amino-terminal region of said allergen.
- 10 3. The variant according to claim 1 or 2, said variant lacking two of said disulfide bridges constituting the structure of said allergen.
- 15 4. The variant according to claim 1, said variant lacking three, or four of said disulfide bridges constituting the structure of said allergen.
5. The variant according to any one of the claims 1 to 4, said variant being a mutein wherein at least one of the cysteines constituting said disulfide bridges is deleted.
- 20 6. The variant according to any one of the claims 1 to 5, said variant being a mutein wherein at least one of the cysteines constituting said disulfide bridges is substituted with an amino acid residue not capable of forming disulfide bridges.
- 25 7. The variant according to claim 6, wherein said amino acid residue is serine or alanine.
8. The variant according to any of claims 1 to 7, wherein said variant has substantially the same length of said allergen.
- 30 9. The variant according to any one of the claims 1 to 8, wherein said allergen is one of the major allergens of Parietaria Judaica.
- 35 10. The variant according to claim 9, wherein said allergen is Parj1, and said cysteines constituting the disulfide bridges are the cysteines 4, 14, 29, 30, 50, 52, 75 and 91.

11. The variant according to claim 10, wherein the sequence of said variant comprises a sequence selected from the group consisting of the sequences reported in the sequence listing as SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 7.

5 12. The variant of anyone of claims 1 to 11 exhibiting reduced IgE binding capability while maintaining the capability of inducing IgG response.

10 13. The variant according to any one of the claims 1 to 12 for use as a medicament or as a diagnostic agent.

15 14. The variant according to claim 13 for use in a patient customised treatment and/or prevention of allergic form associated with an allergen belonging to the family of the ns-LTP proteins.

15 15. The variant according to claim 14 which is a variant of a major allergen of Parietaria Judaica and wherein the allergic form is associated with an allergen of Parietaria Judaica.

20 16. The variant according to claims 14 or 15 for use in the treatment of itch, erythema, edema, wheal, rash (urticaria) formation, rhino-conjunctivitis (seasonal allergies), bronchoconstriction, asthma and anaphylaxis.

25 17. The variant according to claims 13 to 16 for use in preventive specific immunotherapy.

18. The variant according to claim 13 for use as diagnostic agent for patient customised diagnosis of allergic forms.

30 19. A polynucleotide coding for the variant according to any one of the claims 1 to 12.

20. The polynucleotide according to claim 19, wherein said sequence of said variant comprises a sequence selected in the group comprising the sequences reported in the sequence listing as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8.

35 21. A vector comprising at least one polynucleotide according to claim 19 or 20.

22. An use of a variant according to any one of the

claims 1 to 12, for the preparation of a medicament for the treatment and/or the prevention of allergic form associated with an allergen belonging to the family of the ns-LTP proteins.

5 23. The use according to claim 22, wherein said allergen belonging to the family of ns-LTP proteins is the allergen corresponding to said variant.

10 24. The use according to claim 22 or 23, wherein said allergen is a major allergen of Parietaria Judaica and said allergic form associated with said allergen is an allergic form from Parietaria Judaica.

25. A pharmaceutical composition comprising at least one variant according to any one of the claims 1 to 12, and a pharmaceutically acceptable carrier.

15 26. A pharmaceutical composition comprising at least one polynucleotide according to claim 19 or 20, and/or one vector according to claim 21, and a pharmaceutically acceptable carrier.

20 27. A diagnostic agent comprising at least one variant according to any one of the claims 1 to 12, and a pharmaceutically acceptable carrier.

28. A kit for the derivation of a subject-customised allergogram for an allergic form associated with an ns-LTP allergen, comprising

25 - a first composition comprising said ns-LTP allergen in native form together with an acceptable carrier;

30 - at least one composition comprising a single variant of said ns-LTP allergen according to any one of the claims 1 to 12 and an acceptable carrier;

said allergogram being derivable contacting said compositions with immunoglobulins of said subject and observing the effects thus obtained.

35 29. The kit according to claim 28, comprising said first composition and a number of compositions each comprising a single variant of said ns-LTP allergen,

32

equal to the number of the variants of said ns-LTP allergen.

30. The kit according to claim 28 or 29, wherein said ns-LTP allergen is ParJ1.

Parj 1	QETCGTMRV	10	20	25	36	45
	ALMPCLPFVQ	GKEKE	PSKGCCSGAKR	LDGETKTGP	QRVHACECIQT	
Parj 2	EEACGKVVQ	DIMPCLHFVQ	GEEKE	PSKECCSGTKK	LSEEVKTTE	QKREACKCIVR
		alpha 1	loop1	alpha 2	loop2	alpha 3
Parj 1	AMKTYS	56	62	71	88	
	DIDGKLVSE					
Parj 2	ATKGISGI	KNELVAEVP	VPKHCGIVVSKLPPIDV	NMDCK TL		
	Loop3	alpha 4	KKCDIKTTLPPITA	DFDCS KIQTIFRGYY		
			loop4	beta		

Fig. 1

Par j 1 QETCGTMVBA LMPCLPFVQG KEKEPSKGCC SGAKRLDGET KTGPOVRHACECTQ .TAMKT Y .SDIDGKL VDSSLKPIDV NMDCCKTV .
 Soia .PSCP . . . D LSICLNLNLLGG SLG .TYDDCC ALIGGLDIEAVCLCQLRALGI LNLRNRLQLI LN . . . SCGR SYPS NATCPRT
 lyses ..TCGQYTAG LAPCLPYLQG R .GPLGGCC GGVKNLNGSA KTTADRKTACTCLK .SAANA I .KGIDLNKA AGIPSVCKV NIPY .KISP STDCCSTV
 ricco ...NCGQVNKA LSSCUPFLTG FDTTPSLTCC AGYMELKRLA PTVKDKRIACECVK .TAALAR Y .PNIREDAA SSLPYKCGV VINV .PISK TTNCHEI
 tabacco ...SCGQVQSG LAPCLPYLQG R .GPLGSCC GGVKGLGAA KSLSDRKTACTCLK .SAANA I .KGIDMGKA AGLPGACGV NIPY .KISP STDCCSKV
 ricco ...DCGQVNNS LASCLFLTG GVASPASCC AGVQNLKTLA PTSADRRAACECTK .AAAAR F .PTIKQDAA SSLPKKCGV DINI .PISK TTNQAI
 orysa ...TCGQVNNSA VGPCLTYARG G .AGPSAACC SGVRSLKAAA STADRRTACNCLK .NAARG I .KGLNAGNA ASIPSKCGV SVPY .TISA SIDCSRV
 mais ..SCGQVASA IAPCISYARG QGSGPSAGCC SGVRSLNNA RTTADRRAACNCLK .NAAAG V .SGLNAGNA ASIPSKCGV SIPY .TIST STDCSR
 spio1 ..TCGMVSSX LAPCIGYLKG G .PLGGGCC GGIKALNAA ATTPDRKTAACNCLK .SAANA I .KGINYGKA AGLPGMCGV HIPY .AISP STNCNAV
 grano ..DCGHVDSL VRPCLSYVQG G .PGPSGQCC DGVKNLHNQA RSQSDRQSACNCLK .GIARG I .HNLNEDNA RSIPPKCGV NLIPY .TISL NIIDCSR

Fig. 2

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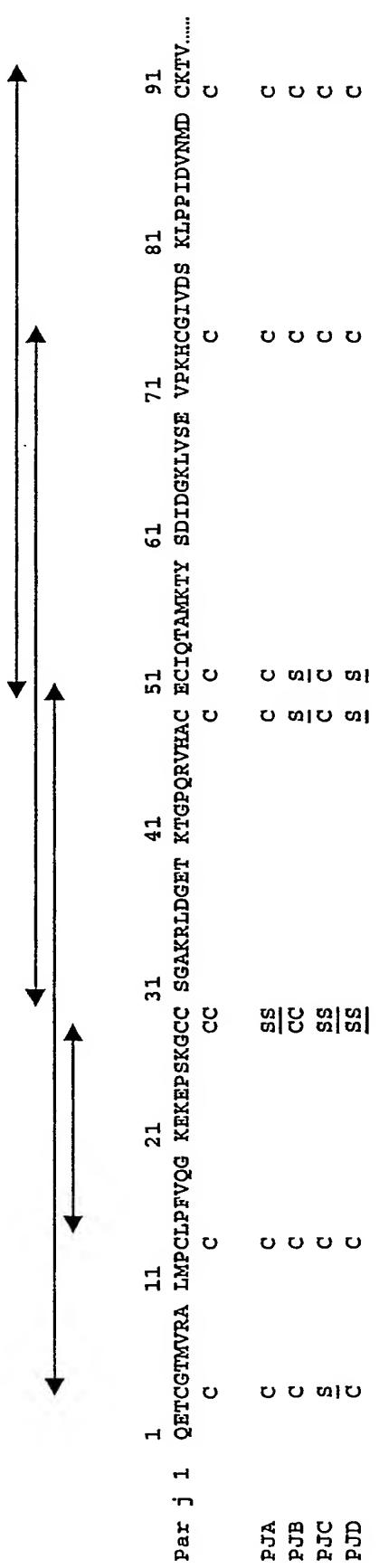


Fig. 3

A

rParj1 PjA PjB PjC PjD

**B**

rParj1 PjA PjB PjC PjD

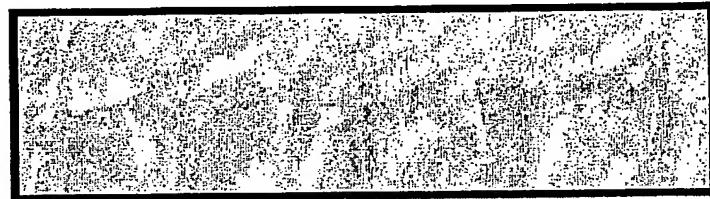


FIG. 4

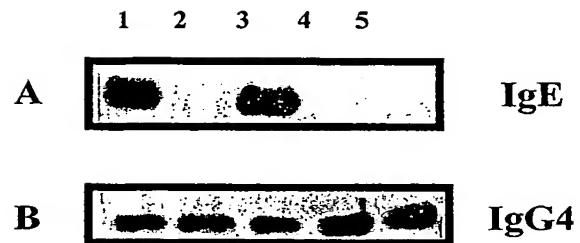


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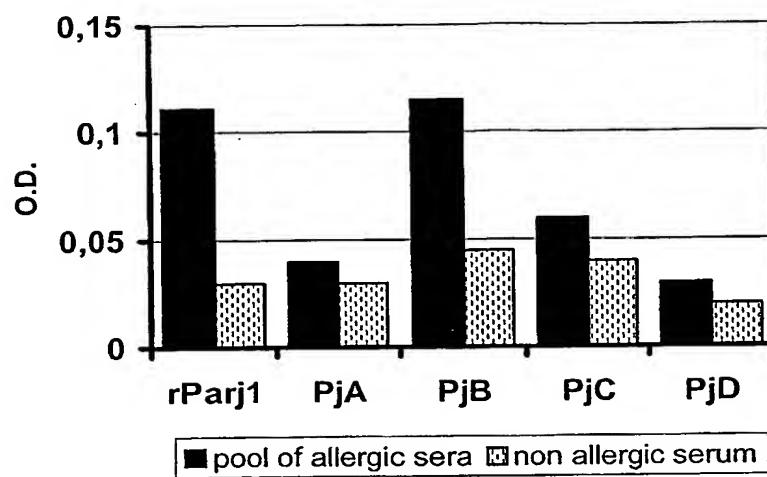


FIG. 6

7/8

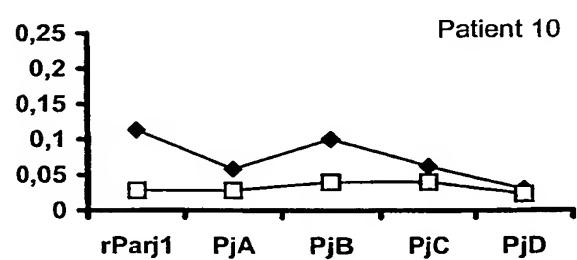
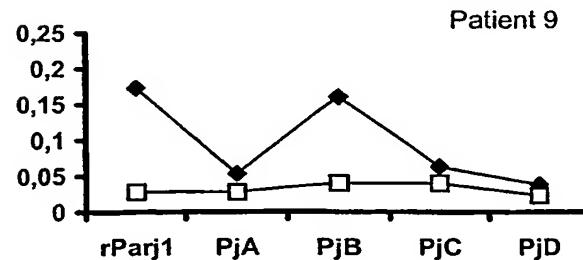
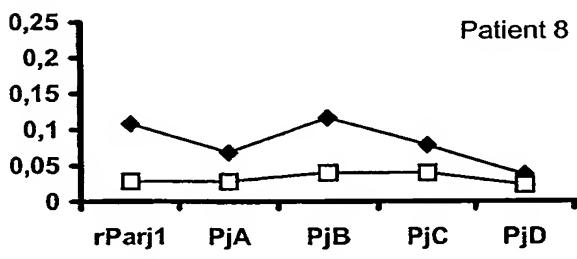
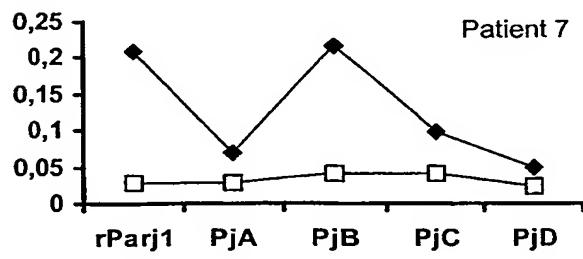
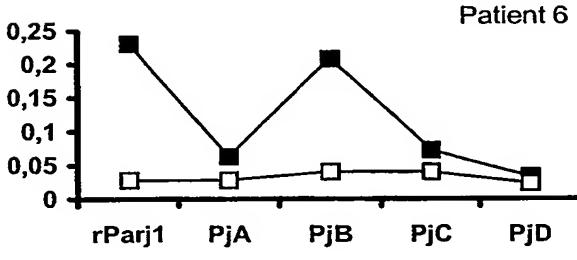
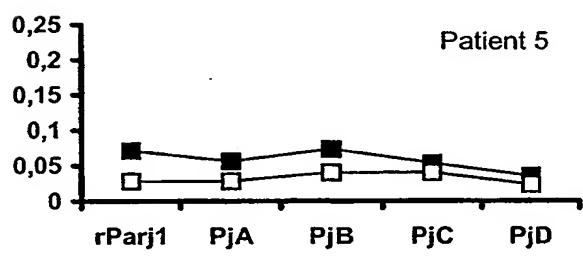
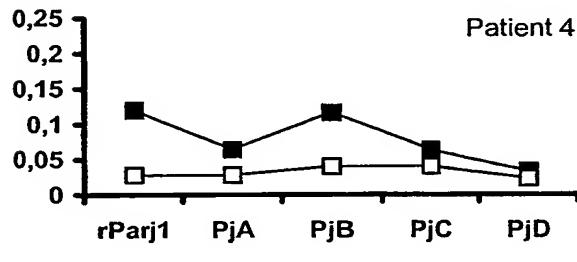
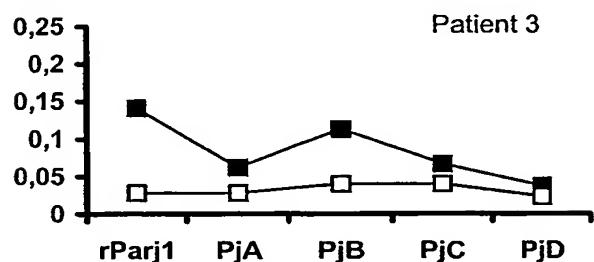
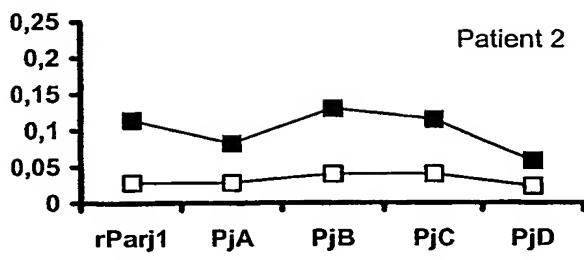
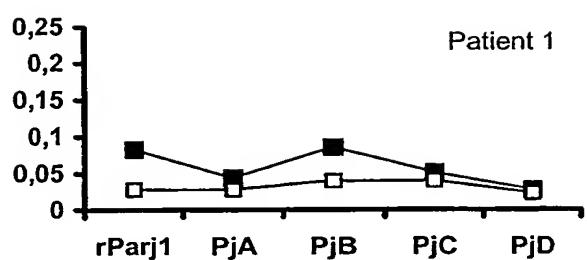


FIG. 7

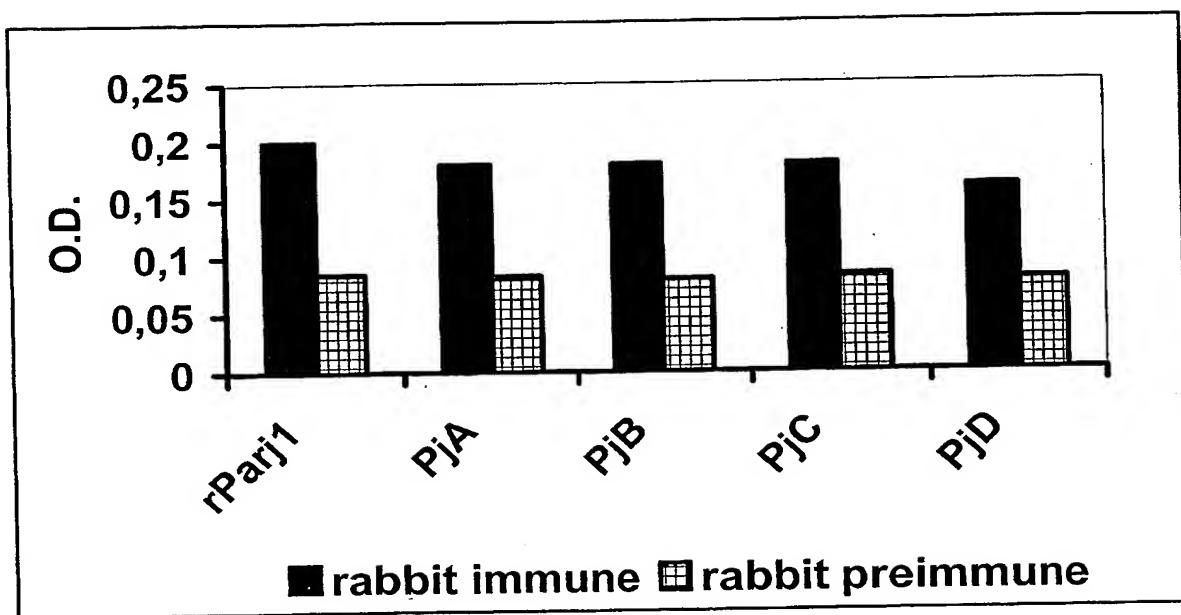


FIG. 8

SEQUENCE LISTING

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10

15

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20

25

30

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40

45

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55

60

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65

70

75

80

aag ctc ccg ccc att gac gtc aac atg gac tgc aag aca gtt gga gtg
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85

90

95

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105

110

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384

Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

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120

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35

40

45

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50

55

60

Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

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70

75

80

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

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90

95

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20 25 30

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50 55 60

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240 Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser
65 70 75 80

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288

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

85 90 95

gtt cct cg^g caa ccc caa ctt cca gtc tct ctc cgt cat ggt ccc gtc
336

Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

100 105 110

acg ggc cca agt gat ccc gcc cac aaa gca cg^g ttg gag aga ccc cag
384

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20 25 30

Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His

35 40 45

Ala Cys Glu Cys Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50

55

60

Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65

70

75

80

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

85

90

95

Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

100

105

110

Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

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125

Ile Arg Val Pro Pro Ala Pro Glu Lys Ala

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135

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of Cys 50 and 52 with Ser

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 96
 Phe Val Gln Gly Lys Glu Lys Glu Pro Ser Lys Gly Cys Cys Ser Gly
 20 25 30

gcc aaa aga ttg gac ggg gag acg aag acg ggg ccg cag agg gtg cac
 144
 Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His
 35 40 45

gct agt gag agc atc cag acc gcc atg aag act tat tcc gac atc gac
 192
 Ala Ser Glu Ser Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp
 50 55 60

ggg aaa ctc gtc agc gag gtc ccc aag cac tgc ggc atc gtt gac agc
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 65 70 75 80

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 Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val
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acg ggc cca agt gat ccc gcc cac aaa gca cgg ttg gag aga ccc cag

384
Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

115

120

125

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15

Phe Val Gln Gly Lys Glu Lys Glu Pro Ser Lys Gly Cys Cys Ser Gly

20

25

30

Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His

35

40

45

Ala Ser Glu Ser Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50

55

60

Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65

70

75

80

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val
85 90 95

Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val
100 105 110

Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln
115 120 125

Ile Arg Val Pro Pro Pro Ala Pro Glu Lys Ala
130 135

<210> 7
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<223> Coding sequence for Parj 1 mutant by substitution of the
Cys 4, 2
9 and 30 with Ser

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48
Gln Glu Thr Ser Gly Thr Met Val Arg Ala Leu Met Pro Cys Leu Pro
1 5 10 15

ttc gtg cag ggg aaa gag aaa gag ccg tca aag ggg agc agc agc ggc
96
Phe Val Gln Gly Lys Glu Lys Glu Pro Ser Lys Gly Ser Ser Ser Gly

20

25

30

gcc aaa aga ttg gac ggg gag acg aag acg ggg ccg cag agg gtg cac
144
Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His

35

40

45

gct tgt gag tgc atc cag acc gcc atg aag act tat tcc gac atc gac
192
Ala Cys Glu Cys Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50

55

60

ggg aaa ctc gtc agc gag gtc ccc aag cac tgc ggc atc gtt gac agc
240
Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65

70

75

80

aag ctc ccg ccc att gac gtc aac atg gac tgc aag aca gtt gga gtg
288
Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

85

90

95

gtt cct cgg caa ccc caa ctt cca gtc tct ctc cgt cat ggt ccc gtc
336
Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

100

105

110

acg ggc cca agt gat ccc gcc cac aaa gca cgg ttg gag aga ccc cag
384
Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

115

120

125

att aga gtt ccg ccc gca ccg gaa aaa gcc taa
420
Ile Arg Val Pro Pro Ala Pro Glu Lys Ala

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<210> 8
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20 25 30

Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His
35 40 45

Ala Cys Glu Cys Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp
50 55 60

Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser
65 70 75 80

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val
85 90 95

Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val
100 105 110

Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

115

120

125

Ile Arg Val Pro Pro Ala Pro Glu Lys Ala
130 135

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<213> Artificial Sequence

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<221> CDS

<222> (1) .. (420)

<223> Coding sequence for Parj 1 mutant by substitution of the
Cys 29,
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Gln Glu Thr Cys Gly Thr Met Val Arg Ala Leu Met Pro Cys Leu Pro

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ttc gtg cag ggg aaa gag aaa gag ccg tca aag ggg agc agc agc ggc
96

Phe Val Gln Gly Lys Glu Lys Glu Pro Ser Lys Gly Ser Ser Ser Gly

20

25

30

gcc aaa aga ttg gac ggg gag acg aag acg ggg ccg cag agg gtg cac
144

Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His

35

40

45

gct agt gag agc atc cag acc gcc atg aag act tat tcc gac atc gac
192

Ala Ser Glu Ser Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50

55

60

ggg aaa ctc gtc agc gag gtc ccc aag cac tgc ggc atc gtt gac agc
240
Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65

70

75

80

aag ctc ccg ccc att gac gtc aac atg gac tgc aag aca gtt gga gtg
288
Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

85

90

95

gtt cct cgg caa ccc caa ctt cca gtc tct ctc cgt cat ggt ccc gtc
336
Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

100

105

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acg ggc cca agt gat ccc gcc cac aaa gca cgg ttg gag aga ccc cag
384
Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

115

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Ile Arg Val Pro Pro Ala Pro Glu Lys Ala

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Ala Lys Arg Leu Asp Gly Glu Thr Lys Thr Gly Pro Gln Arg Val His

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Ala Ser Glu Ser Ile Gln Thr Ala Met Lys Thr Tyr Ser Asp Ile Asp

50

55

60

Gly Lys Leu Val Ser Glu Val Pro Lys His Cys Gly Ile Val Asp Ser

65

70

75

80

Lys Leu Pro Pro Ile Asp Val Asn Met Asp Cys Lys Thr Val Gly Val

85

90

95

Val Pro Arg Gln Pro Gln Leu Pro Val Ser Leu Arg His Gly Pro Val

100

105

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Thr Gly Pro Ser Asp Pro Ala His Lys Ala Arg Leu Glu Arg Pro Gln

115

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<210> 11

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<220>
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<222> (10)..(21)
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<220>
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<222> (4)..(9)
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<210> 12
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<223> Sequence mapping from nucleotide 405 to nucleotide 423 o
f Parj1.0
102

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<210> 13

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02

<220>
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j1.0102

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<222> (6)..(6)
<223> Residue mutated with respect to the corresponding position in Par
j1.0102

<400> 13
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<210> 14
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<223> Restriction site for PstI enzyme

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<223> Residue mutated with respect to the corresponding position in Par
      j1.0102

<220>
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<223> Residue mutated with respect to the corresponding position in Par
      j1.0102

<400> 14
ggtgctgcag cggcgccaaa agattggacg gggagacgaa gacggggccg cagagggt
gc       60

acgctagtga gagcatc
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<210> 15
<211> 26
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      j1.0102

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<222> (19)..(19)
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j1.0102

<400> 15
gcagtagatccc aagaaaaccag cgggac
26

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IT 01/00471

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/28 C12N15/70 C07K14/415 A61K39/36 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLOMBO P. ET AL: "Identification of an immunodominant IgE epitope of the <i>Parietaria judaica</i> major allergen" JOURNAL OF IMMUNOLOGY, vol. 160, no. 6, 15 March 1998 (1998-03-15), pages 2780-5, XP002186915 abstract	1,2,5-26
Y	see above page 2784; claims 1,2 ---	3,4, 27-30 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 January 2002	25/01/2002

Name and mailing address of the ISA
 European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
 Fax (+31-70) 340-3016

Authorized officer

Celler, J

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IT 01/00471

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FERREIRA F. ET AL: "Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy" FASEB J, vol. 12, February 1998 (1998-02), pages 231-242, XP002186916 page 232, column 1 page 237, column 1 -page 238, column 2 page 240, column 1 -column 2 ----	1-30
Y	OLSSON S. ET AL : "Contribution of disulphide bonds to antigenicity of Lep d 2, the major allergen of the dust mite Lepidoglyphus destructor" MOLECULAR IMMUNOLOGY, vol. 35, 1998, pages 1017-1023, XP001026605 abstract page 1021, column 2 -page 1022, column 1 ----	3,4, 27-30
A	WO 00 44781 A (UNIV CALIFORNIA) 3 August 2000 (2000-08-03) abstract page 1 -page 2 page 17 ----	1-30
P,X	BONURA A. ET AL: "Hypoallergenic variants of the Parietaria judaica makor allergen Par j 1: a member of the non-specific lipid transfer protein plant family" INT ARCH ALLERGY IMMUNOL, vol. 126, no. 1, September 2001 (2001-09), pages 32-40, XP001037388 the whole document -----	1-30

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 19-21(partly)

Present claims 19-21 relate to an extremely large number of possible polynucleotides. In fact, due to the degeneracy of the genetic code the claims contain so many possible permutations that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a complete search over the whole scope of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely polynucleotide sequences reported in the present sequence listing as Seq. ID. No.: 2, 4, 6 and 8.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No

PCT/IT 01/00471

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
WO 0044781	A 03-08-2000	AU EP WO	2738300 A 1147131 A1 0044781 A1	18-08-2000 24-10-2001 03-08-2000

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